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Invention: PRODUCTION OF HYDROXYLATED FATTY ACIDS IN GENETICALLY MODIFIED PLANTS

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This is a:

- ☐ Provisional Application
- ☐ Regular Utility Application
- ☒ Continuing Application
 - ☒ The contents of the parent are incorporated by reference
- ☐ PCT National Phase Application
- ☐ Design Application
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SPECIFICATION

PRODUCTION OF HYDROXYLATED FATTY ACIDS
IN GENETICALLY MODIFIED PLANTS

5 CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of
U.S. patent application Serial No. 08/530,862,
filed September 20, 1995, the entire contents of
which are hereby incorporated by reference and
10 relied upon.

GOVERNMENT RIGHTS

The invention described herein was made in
the course of work under grant number DE-FG02-
15 94ER20133 from the U.S. Department of Energy and
grant No. MCB9305269 from the National Science
Foundation. Therefore, the U.S. Government has
certain rights under this invention.

20 TECHNICAL FIELD

The present invention concerns the
identification of nucleic acid sequences and
constructs, and methods related thereto, and the
use of these sequences and constructs to produce
25 genetically modified plants for the purpose of
altering the fatty acid composition of plant oils,
waxes and related compounds.

DEFINITIONS

The subject of this invention is a class of enzymes that introduce a hydroxyl group into several different fatty acids resulting in the production of several different kinds of hydroxylated fatty acids. In particular, these enzymes catalyze hydroxylation of oleic acid to 12-hydroxy oleic acid and icosenoic acid to 14-hydroxy icosenoic acid. Other fatty acids such as palmitoleic and erucic acids may also be substrates. Since it is not possible to refer to the enzyme by reference to a unique substrate or product, we refer to the enzyme throughout as kappa hydroxylase to indicate that the enzyme introduces the hydroxyl three carbons distal (i.e., away from the carboxyl carbon of the acyl chain) from a double bond located near the center of the acyl chain.

The following fatty acids are also the subject of this invention: ricinoleic acid, 12-hydroxyoctadec-*cis*-9-enoic acid (12OH-18:1^{cisΔ⁹}); lesquerolic acid, 14-hydroxy-*cis*-11-icosenoic acid (14OH-20:1^{cisΔ¹¹}); densipolic acid, 12-hydroxyoctadec-*cis*-9,15-dienoic acid (12OH-18:2^{cisΔ^{9,15}}); auricollic acid, 14-hydroxy-*cis*-11,17-icosadienoic acid (14OH-20:2^{cisΔ^{11,17}}); hydroxyerucic, 16-hydroxydocos-*cis*-13-enoic acid (16OH-22:1^{cisΔ¹³}); hydroxypalmitoleic, 12-

hydroxyhexadec-*cis*-9-enoic (12OH-16:1^{cisΔ9});
icosenoic acid (20:1^{cisΔ11}). It will be noted
that icosenoic acid is spelled eicosenoic acid in
some countries.

5

BACKGROUND

Extensive surveys of the fatty acid
composition of seed oils from different species of
higher plants have resulted in the identification
10 of at least 33 structurally distinct
monohydroxylated plant fatty acids, and 12
different polyhydroxylated fatty acids that are
accumulated by one or more plant species (reviewed
by van de Loo et al. 1993). Ricinoleic acid, the
15 principal constituent of the seed oil from the
castor plant *Ricinus communis* (L.), is of
commercial importance. We have previously
described the cloning of a gene from this species
that encodes a fatty acid hydroxylase, and the use
20 of this gene to produce ricinoleic acid in
transgenic plants of other species (see U.S.
patent application Serial No. 08/320,982, filed
October 11, 1994). The scientific evidence
supporting the claims in that patent application
25 were subsequently published (van de Loo et al.,
1995).

The use of the castor hydroxylase gene to
also produce other hydroxylated fatty acids such

as lesquerolic acid, densipolic acid,
hydroxypalmitoleic, hydroxyerucic and auricolic
acid in transgenic plants is the subject of this
invention. In addition, the identification of a
5 gene encoding a homologous hydroxylase from
Lesquerella fendleri, and the use of this gene to
produce these hydroxylated fatty acids in
transgenic plants is the subject of this
invention.

10 Castor is a minor oilseed crop.
Approximately 50% of the seed weight is oil
(triacylglycerol) in which 85-90% of total fatty
acids are the hydroxylated fatty acid, ricinoleic
acid. Oil pressed or extracted from castor seeds
15 has many industrial uses based upon the properties
endowed by the hydroxylated fatty acid. The most
important uses are production of paints and
varnishes, nylon-type synthetic polymers, resins,
lubricants, and cosmetics (Atsmon 1989).

20 In addition to oil, the castor seed contains
the extremely toxic protein ricin, allergenic
proteins, and the alkaloid ricinine. These
constituents preclude the use of the untreated
seed meal (following oil extraction) as a
25 livestock feed, normally an important economic
aspect of oilseed utilization. Furthermore, with
the variable nature of castor plants and a lack of
investment in breeding, castor has few favorable

agronomic characteristics.

For a combination of these reasons, castor is no longer grown in the United States and the development of an alternative domestic source of hydroxylated fatty acids would be attractive. The production of ricinoleic acid, the important constituent of castor oil, in an established oilseed crop through genetic engineering would be a particularly effective means of creating a domestic source.

Because there is no practical source of lesquerolic, densipolic and auricollic acids from plants that are adapted to modern agricultural practices, there is currently no large-scale use of these fatty acids by industry. However, the fatty acids would have uses similar to those of ricinoleic acid if they could be produced in large quantities at comparable cost to other plant-derived fatty acids (Smith 1985).

Plant species, such as certain species in the genus *Lesquerella*, that accumulate a high proportion of these fatty acids, have not been domesticated and are not currently considered a practical source of fatty acids (Hirsinger, 1989). This invention represents a useful step toward the eventual production of these and other hydroxylated fatty acids in transgenic plants of agricultural importance.

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. . . The taxonomic relationships between plants having similar or identical kinds of unusual fatty acids have been examined (van de Loo et al., 1993). In some cases, particular fatty acids occur mostly or solely in related taxa. In other cases there does not appear to be a direct link between taxonomic relationships and the occurrence of unusual fatty acids. In this respect, ricinoleic acid has now been identified in 12 genera from 10 families (reviewed in van de Loo et al., 1993). Thus, it appears that the ability to synthesize hydroxylated fatty acids has evolved several times independently during the radiation of the angiosperms. This suggested to us that the enzymes which introduce hydroxyl groups into fatty acids arose by minor modifications of a related enzyme.

Indeed, as shown herein, the sequence similarity between $\Delta 12$ fatty acid desaturases and the kappa hydroxylase from castor is so high that it is not possible to unambiguously determine whether a particular enzyme is a desaturase or a hydroxylase on the basis of evidence in the scientific literature. Similarly, a patent application (PCT/US93/09987) that purports to teach the isolation and use of $\Delta 12$ fatty acid desaturases does not teach how to distinguish a hydroxylase from a desaturase. In view of the

importance of being able to distinguish between these activities for the purpose of genetic engineering of plant oils, the utility of that application is limited to the several instances where direct experimental evidence (e.g., altered fatty acid composition in transgenic plants) was presented to support the assignment of function. A method for distinguishing between fatty acid desaturases and fatty acid hydroxylases on the basis of amino acid sequence of the enzyme is also a subject of this invention.

A feature of hydroxylated or other unusual fatty acids is that they are generally confined to seed triacylglycerols, being largely excluded from the polar lipids by unknown mechanisms (Battey and Ohlrogge 1989; Prasad et al., 1987). This is particularly intriguing since diacylglycerol is a precursor of both triacylglycerol and polar lipid. With castor microsomes, there is some evidence that the pool of ricinoleoyl-containing polar lipid is minimized by a preference of diacylglycerol acyltransferase for ricinoleate-containing diacylglycerols (Bafor et al. 1991). Analyses of vegetative tissues have generated few reports of unusual fatty acids, other than those occurring in the cuticle. The cuticle contains various hydroxylated fatty acids which are interesterified to produce a high molecular weight

polyester which serves a structural role. A small number of other exceptions exist in which unusual fatty acids are found in tissues other than the seed.

5 The biosynthesis of ricinoleic acid from oleic acid in the developing endosperm of castor (*Ricinus communis*) has been studied by a variety of methods. Morris (1967) established in double-labeling studies that hydroxylation occurs
10 directly by hydroxyl substitution rather than via an unsaturated-, keto- or epoxy-intermediate. Hydroxylation using oleoyl-CoA as precursor can be demonstrated in crude preparations or microsomes, but activity in microsomes is unstable and
15 variable, and isolation of the microsomes involved a considerable, or sometimes complete loss of activity (Galliard and Stumpf, 1966; Moreau and Stumpf, 1981). Oleic acid can replace oleoyl-CoA as a precursor, but only in the presence of CoA,
20 Mg^{2+} and ATP (Galliard and Stumpf, 1966) indicating that activation to the acyl-CoA is necessary. However, no radioactivity could be detected in ricinoleoyl-CoA (Moreau and Stumpf, 1981). These and more recent observations (Bafor
25 et al., 1991) have been interpreted as evidence that the substrate for the castor oleate hydroxylase is oleic acid esterified to phosphatidylcholine or another phospholipid.

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The hydroxylase is sensitive to cyanide and azide, and dialysis against metal chelators reduces activity, which could be restored by addition of FeSO_4 , suggesting iron involvement in enzyme activity (Galliard and Stumpf, 1966). Ricinoleic acid synthesis requires molecular oxygen (Galliard and Stumpf, 1966; Moreau and Stumpf 1981) and requires NAD(P)H to reduce cytochrome b5 which is thought to be the intermediate electron donor for the hydroxylase reaction (Smith et al., 1992). Carbon monoxide does not inhibit hydroxylation, indicating that a cytochrome P450 is not involved (Galliard and Stumpf, 1966; Moreau and Stumpf 1981). Data from a study of the substrate specificity of the hydroxylase show that all substrate parameters (i.e., chain length and double bond position with respect to both ends) are important; deviations in these parameters caused reduced activity relative to oleic acid (Howling et al., 1972). The position at which the hydroxyl was introduced, however, was determined by the position of the double bond, always being three carbons distal. Thus, the castor acyl hydroxylase enzyme can produce a family of different hydroxylated fatty acids depending on the availability of substrates. Thus, as a matter of convenience, we refer to the enzyme throughout as a kappa hydroxylase (rather

than an oleate hydroxylase) to indicate the broad substrate specificity.

The castor kappa hydroxylase has many superficial similarities to the microsomal fatty acyl desaturases (Browse and Somerville, 1991).

In particular, plants have a microsomal oleate desaturase active at the $\Delta 12$ position. The substrate of this enzyme (Schmidt et al., 1993) and of the hydroxylase (Bafor et al., 1991)

appears to be a fatty acid esterified to the *sn*-2 position of phosphatidylcholine. When oleate is the substrate, the modification occurs at the same position ($\Delta 12$) in the carbon chain, and requires the same cofactors, namely electrons from NADH via cytochrome *b*₅ and molecular oxygen. Neither enzyme is inhibited by carbon monoxide (Moreau and Stumpf, 1981), the characteristic inhibitor of cytochrome P450 enzymes.

There do not appear to have been any published biochemical studies of the properties of the hydroxylase enzyme(s) in *Lesquerella*.

Conceptual basis of the invention

In U.S. patent application No. 08/320,982, we described the use of a cDNA clone from castor for the production of ricinoleic acid in transgenic plants. As noted above, biochemical studies by others had suggested that the castor hydroxylase

may not have strict specificity for oleic acid but would also catalyze hydroxylation of other fatty acids such as icosenoic acid (20:1^{cisΔ11}) (Howling et al., 1972). Based on these studies, our previous application No. 08/320,982 noted in Example 2 that the expression of the castor hydroxylase in transgenic plants of species such as *Brassica napus* and *Arabidopsis thaliana* that accumulate fatty acids such as icosenoic acid (20:1^{cisΔ11}) and erucic acid (13-docosenoic acid; 22:1^{cisΔ13}) would be expected to accumulate some of the hydroxylated derivatives of these fatty acids due to the activity of the hydroxylase on these fatty acids. We have now obtained additional direct evidence for such a claim based on the production of ricinoleic, lesquerolic, densipolic and auricolic fatty acids in transgenic *Arabidopsis* plants and have included such evidence herein as Example 1.

In Example 3 of the previous application, we taught the various methods by which the castor hydroxylase clone and sequences derived thereof could be used to identify other hydroxylase clones from plant species such as *Lesquerella fendleri* that are known to accumulate hydroxylated fatty acids in seed oils. In this continuation we have provided an example of the use of that aspect of the invention for the isolation of a novel

- hydroxylase gene from *Lesquerella fendleri*.

In view of the high degree of sequence similarity between $\Delta 12$ fatty acid desaturases and the castor hydroxylase (van de Loo et al., 1995), the validity of claims (e.g., PCT WO 94/11516) for the use of desaturase or hydroxylase genes or sequences derived therefrom for the identification of genes of identical function from other species must be viewed with skepticism. In this application, we teach a method by which hydroxylase genes can be distinguished from desaturases and describe methods by which $\Delta 12$ desaturases can be converted to hydroxylases by the modification of the gene encoding the desaturases. A mechanistic basis for the similar reaction mechanisms of desaturases and hydroxylases was presented in the earlier patent application (No. 08/320,982). Briefly, the available evidence suggests that fatty acid desaturases have a similar reaction mechanism to the bacterial enzyme methane monooxygenase which catalyses a reaction involving oxygen-atom transfer ($\text{CH}_4 \rightarrow \text{CH}_3\text{OH}$) (van de Loo et al., 1993). The cofactor in the hydroxylase component of methane monooxygenase is termed a μ -oxo bridged diiron cluster (FeOFe). The two iron atoms of the FeOFe cluster are liganded by protein-derived nitrogen or oxygen atoms, and are tightly redox-

coupled by the covalently-bridging oxygen atom.
The FeOFe cluster accepts two electrons, reducing
it to the diferrous state, before oxygen binding.
Upon oxygen binding, it is likely that heterolytic
5 cleavage also occurs, leading to a high valent
oxoiron reactive species that is stabilized by
resonance rearrangements possible within the
tightly coupled FeOFe cluster. The stabilized
high-valent oxoiron state of methane monooxygenase
10 is capable of proton extraction from methane,
followed by oxygen transfer, giving methanol. The
FeOFe cofactor has been shown to be directly
relevant to plant fatty acid modifications by the
demonstration that castor stearoyl-ACP desaturase
15 contains this type of cofactor (Fox et al., 1993).

On the basis of the foregoing considerations,
we hypothesized that the castor oleate hydroxylase
is a structurally modified fatty acyl desaturase,
based upon three arguments. The first argument
20 involves the taxonomic distribution of plants
containing ricinoleic acid. Ricinoleic acid has
been found in 12 genera of 10 families of higher
plants (reviewed in van de Loo et al., 1993).
Thus, plants in which ricinoleic acid occurs are
25 found throughout the plant kingdom, yet close
relatives of these plants do not contain the
unusual fatty acid. This pattern suggests that
the ability to synthesize ricinoleic acid has

arisen (and been lost) several times
independently, and is therefore a quite recent
divergence. In other words, the ability to
synthesize ricinoleic acid has evolved rapidly,
5 suggesting that a relatively minor genetic change
in the structure of the ancestral enzyme was
necessary to accomplish it.

The second argument is that many biochemical
properties of castor kappa hydroxylase are similar
10 to those of the microsomal desaturases, as
discussed above (e.g., both preferentially act on
fatty acids esterified to the sn-2 position of
phosphatidylcholine, both use cytochrome b5 as an
intermediate electron donor, both are inhibited by
15 cyanide, both require molecular oxygen as a
substrate, both are thought to be located in the
endoplasmic reticulum).

The third argument stems from the discussion
of oxygenase cofactors above, in which it is
20 suggested that the plant membrane bound fatty acid
desaturases may have a μ -oxo bridged diiron
cluster-type cofactor, and that such cofactors are
capable of catalyzing both fatty acid
desaturations and hydroxylations, depending upon
25 the electronic and structural properties of the
protein active site.

Taking these three arguments together, it was
hypothesized that kappa hydroxylase of castor

endosperm is homologous to the microsomal oleate
Δ12 desaturase found in all plants. The evidence
supporting this hypothesis was disclosed in the
previous patent application (No. 08/320,982). A
5 number of genes encoding microsomal Δ12
desaturases from various species have recently
been cloned (Okuley et al., 1994) and substantial
information about the structure of these enzymes
is now known (Shanklin et al. 1994). Hence, in
10 the following invention we teach how to use
structural information about fatty acyl
desaturases to isolate kappa hydroxylase genes of
this invention. This example teaches the method
by which any carbon-monoxide insensitive plant
15 fatty acyl hydroxylase gene can be identified by
one skilled in the art.

An unpredicted outcome of our studies on the
castor hydroxylase gene in transgenic Arabidopsis
plants was the discovery that expression of the
20 hydroxylase leads to increased accumulation of
oleic acid in seed lipids. Because of the low
nucleotide sequence homology between the castor
hydroxylase and the Δ12-desaturase (about 67%), we
consider it unlikely that this effect is due to
25 silencing (also called sense-suppression or
cosuppression) of the expression of the desaturase
gene by the hydroxylase gene. Whatever the basis
for the effect, this invention teaches the use of

Hydroxylase genes to alter the level of fatty acid unsaturation in transgenic plants. On the basis of a hypothesis about the mechanisms of the effect, this invention also teaches the use of genetically modified hydroxylase and desaturase genes to achieve directed modification of fatty acid unsaturation levels.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-D show the mass spectra of hydroxy fatty acids standards (Figure 1A, O-TMS-methylricinoleate; Figure 1B, O-TMS-methyl densipoleate; Figure 1C, O-TMS-methyl lesqueroleate; and Figure 1D, O-TMS-methylauricoleate).

Figure 2 shows the fragmentation pattern of trimethylsilylated methyl esters of hydroxy fatty acids.

Figure 3A shows the gas chromatogram of fatty acids extracted from seeds of wild type Arabidopsis plants. Figure 3B shows the gas chromatogram of fatty acids extracted from seeds of transgenic Arabidopsis plants containing the fah12 hydroxylase gene. The numbers indicate the following fatty acids: [1] 16:0; [2] 18:0; [3] 18:1cis Δ 9; [4] 18:2cis Δ 9,12; [5] 20:0; [6] 20:1cis Δ 11; [7] 18:3cis Δ 9,12,15; [8] 22:1cis Δ 13; [9]

24:1^{cis}Δ¹³; [10]ricinoleic acid; [11] densipolic acid; [12] lesquerolic acid; [13] auricolic acid.

Figures 4A-D show the mass spectra of novel fatty acids found in seeds of transgenic plants.

5 Figure 4A shows the mass spectrum of peak 10 from Figure 3B. Figure 4B shows the mass spectrum of peak 11 from Figure 3B. Figure 4C shows the mass spectrum of peak 12 from Figure 3B. Figure 4D shows the mass spectrum of peak 13 from Figure 3B.

10 Figure 5 shows the nucleotide sequence of pLesq2 (SEQ ID NO:1).

Figure 6 shows the nucleotide sequence of pLesq3 (SEQ ID NO:2).

15 Figure 7 shows a Northern blot of total RNA from seeds of *L. fendleri* probed with pLesq2 or pLesq3. S, indicates RNA is from seeds; L, indicates RNA is from leaves.

20 Figures 8A-B show the nucleotide sequence of genomic clone encoding pLesq-HYD (SEQ ID NO:3), and the deduced amino acid sequence of hydroxylase enzyme encoded by the gene (SEQ ID NO:4).

Figures 9A-B show multiple sequence alignment of deduced amino acid sequences for kappa hydroxylases and microsomal Δ¹² desaturases.

25 Abbreviations are: Rcfah12, fah12 hydroxylase gene from *R. communis* (van de Loo et al., 1995); Lffah12, kappa hydroxylase gene from *L. fendleri*; Atfad2, fad2 desaturase from *Arabidopsis thaliana*

• (Okuley et al., 1994); Gmfad2-1, fad2 desaturase
from *Glycine max* (GenBank accession number
L43920); Gmfad2-2, fad2 desaturase from *Glycine*
max (Genbank accession number L43921); Zmfad2,
5 fad2 desaturase from *Zea mays* (PCT/US93/09987);
Rcfad2, fragment of fad2 desaturase from *R.*
communis (PCT/US93/09987); Bnfad2, fad2 desaturase
from *Brassica napus* (PCT/US93/09987); LFFAH12.AMI,
SEQ ID NO:4; FAH12.AMI, SEQ ID NO:5; ATFAD2.AMI,
10 SEQ ID NO:6; BNFAD2.AMI, SEQ ID NO:7; GMFAD2-
1.AMI, SEQ ID NO:8; GMFAD2-2.AMI, SEQ ID NO:9;
ZMFAD2.AMI, SEQ ID NO:10; and RCFAD2.AMI, SEQ ID
NO:11.

Figure 10 shows a Southern blot of genomic
15 DNA from *L. fendleri* probed with pLesq-HYD.
E=EcoRI, H = HindIII, X = XbaI.

Figure 11 shows a map of binary Ti plasmid
pSLJ44024.

Figure 12 shows a map of plasmid pYES2.0

20 Figure 13 shows part of a gas chromatogram of
derivatized fatty acids from yeast cells that
contain plasmid pLesqYes in which expression of
the hydroxylase gene was induced by addition of
galactose to the growth medium. The arrow points
25 to a peak that is not present in uninduced cells.
The lower part of the figure is the mass spectrum
of the peak indicated by the arrow.

SUMMARY OF THE INVENTION

This invention relates to plant fatty acyl hydroxylases. Methods to use conserved amino acid or nucleotide sequences to obtain plant fatty acyl hydroxylases are described. Also described is the use of cDNA clones encoding a plant hydroxylase to produce a family of hydroxylated fatty acids in transgenic plants.

In a first embodiment, this invention is directed to recombinant DNA constructs which can provide for the transcription or transcription and translation (expression) of the plant kappa hydroxylase sequence. In particular, constructs which are capable of transcription or transcription and translation in plant host cells are preferred. Such constructs may contain a variety of regulatory regions including transcriptional initiation regions obtained from genes preferentially expressed in plant seed tissue. In a second aspect, this invention relates to the presence of such constructs in host cells, especially plant host cells which have an expressed plant kappa hydroxylase therein.

In yet another aspect, this invention relates to a method for producing a plant kappa hydroxylase in a host cell or progeny thereof via the expression of a construct in the cell. Cells

containing a plant kappa hydroxylase as a result of the production of the plant kappa hydroxylase encoding sequence are also contemplated herein.

In another embodiment, this invention relates to methods of using a DNA sequence encoding a plant kappa hydroxylase for the modification of the proportion of hydroxylated fatty acids produced within a cell, especially plant cells. Plant cells having such a modified hydroxylated fatty acid composition are also contemplated herein.

In a further aspect of this invention, plant kappa hydroxylase proteins and sequences which are related thereto, including amino acid and nucleic acid sequences, are contemplated. Plant kappa hydroxylase exemplified herein includes a *Lesquerella fendleri* fatty acid hydroxylase. This exemplified fatty acid hydroxylase may be used to obtain other plant fatty acid hydroxylases of this invention.

In a further aspect of this invention, a nucleic acid sequence which directs the seed specific expression of an associated polypeptide coding sequence is described. The use of this nucleic acid sequence or fragments derived thereof, to obtain seed-specific expression in higher plants of any coding sequence is contemplated herein.

In a further aspect of this invention, the use of genes encoding fatty acyl hydroxylases of this invention are used to alter the amount of fatty acid unsaturation of seed lipids. We further envision the use of genetically modified hydroxylase and desaturase genes to achieve directed modification of fatty acid unsaturation levels.

10 DETAILED DESCRIPTION OF THE INVENTION

A genetically transformed plant of the present invention which accumulates hydroxylated fatty acids can be obtained by expressing the double-stranded DNA molecules described in this application.

A plant fatty acid hydroxylase of this invention includes any sequence of amino acids, such as a protein, polypeptide or peptide fragment, or nucleic acid sequences encoding such polypeptides, obtainable from a plant source which demonstrates the ability to catalyze the production of ricinoleic, lesquerolic, hydroxyerucic (16-hydroxydocos-*cis*-13-enoic acid) or hydroxypalmitoleic (12-hydroxyhexadec-*cis*-9-enoic) from CoA, ACP or lipid-linked monoenoic fatty acid substrates under plant enzyme reactive conditions. By "enzyme reactive conditions" is

meant that any necessary conditions are available in an environment (i.e., such factors as temperature, pH, lack of inhibiting substances) which will permit the enzyme to function.

5 Preferential activity of a plant fatty acid hydroxylase toward a particular fatty acyl substrate is determined upon comparison of hydroxylated fatty acid product amounts obtained per different fatty acyl substrates. For example, 10 by "oleate preferring" is meant that the hydroxylase activity of the enzyme preparation demonstrates a preference for oleate-containing substrates over other substrates. Although the precise substrate of the castor fatty acid 15 hydroxylase is not known, it is thought to be a monounsaturated fatty acid moiety which is esterified to a phospholipid such as phosphatidylcholine. However, it is also possible that monounsaturated fatty acids esterified to 20 phosphatidylethanolamine, phosphatidic acid or a neutral lipid such as diacylglycerol or a Coenzyme-A thioester may also be substrates.

As noted above, significant activity has been observed in radioactive labelling studies using 25 fatty acyl substrates other than oleate (Howling et al., 1972) indicating that the substrate specificity is for a family of related fatty acyl compounds. Because the castor hydroxylase

introduces hydroxy groups three carbons from a double bond, proximal to the methyl carbon of the fatty acid, we term the enzyme a kappa hydroxylase for convenience. Of particular interest, we envision that the castor kappa hydroxylase may be used for production of 12-hydroxy-9-octadecenoic acid (ricinoleate), 12-hydroxy-9-hexadecenoic acid, 14-hydroxy-11-eicosenoic acid, 16-hydroxy-13-docosanoic acid, 9-hydroxy-6-octadecenoic acid by expression in plants species which produce the non-hydroxylated precursors. We also envision production of additionally modified fatty acids such as 12-hydroxy-9,15-octadecadienoic acid that result from desaturation of hydroxylated fatty acids (e.g., 12-hydroxy-9-octadecenoic acid in this example).

We also envision that future advances in the genetic engineering of plants will lead to production of substrate fatty acids, such as icosenoic acid esters, and palmitoleic acid esters in plants that do not normally accumulate such fatty acids. We envision that the invention described herein may be used in conjunction with such future improvements to produce hydroxylated fatty acids of this invention in any plant species that is amenable to directed genetic modification. Thus, the applicability of this invention is not limited in our conception only to those species

that currently accumulate suitable substrates.

As noted above, a plant kappa hydroxylase of this invention will display activity towards various fatty acyl substrates. During biosynthesis of lipids in a plant cell, fatty acids are typically covalently bound to acyl carrier protein (ACP), coenzyme A (CoA) or various cellular lipids. Plant kappa hydroxylases which display preferential activity toward lipid-linked acyl substrate are especially preferred because they are likely to be closely associated with normal pathway of storage lipid synthesis in immature embryos. However, activity toward acyl-CoA substrates or other synthetic substrates, for example, is also contemplated herein.

Other plant kappa hydroxylases are obtainable from the specific exemplified sequences provided herein. Furthermore, it will be apparent that one can obtain natural and synthetic plant kappa hydroxylases including modified amino acid sequences and starting materials for synthetic-protein modeling from the exemplified plant kappa hydroxylase and from plant kappa hydroxylases which are obtained through the use of such exemplified sequences. Modified amino acid sequences include sequences which have been mutated, truncated, increased and the like, whether such sequences were partially or wholly

synthesized. Sequences which are actually purified from plant preparations or are identical or encode identical proteins thereto, regardless of the method used to obtain the protein or sequence, are equally considered naturally derived.

Thus, one skilled in the art will readily recognize that antibody preparations, nucleic acid probes (DNA and RNA) and the like may be prepared and used to screen and recover "homologous" or "related" kappa hydroxylases from a variety of plant sources. Typically, nucleic acid probes are labeled to allow detection, preferably with radioactivity although enzymes or other methods may also be used. For immunological screening methods, antibody preparations either monoclonal or polyclonal are utilized. Polyclonal antibodies, although less specific, typically are more useful in gene isolation. For detection, the antibody is labeled using radioactivity or any one of a variety of second antibody/enzyme conjugate systems that are commercially available.

Homologous sequences are found when there is an identity of sequence and may be determined upon comparison of sequence information, nucleic acid or amino acid, or through hybridization reactions between a known kappa hydroxylase and a candidate source. Conservative changes, such as Glu/Asp,

Val/Ile, Ser/Thr, Arg/Lys and Gln/Asn may also be considered in determining sequence homology. Typically, a lengthy nucleic acid sequence may show as little as 50-60% sequence identity, and more preferably at least about 70% sequence identity, between the target sequence and the given plant kappa hydroxylase of interest excluding any deletions which may be present, and still be considered related. Amino acid sequences are considered homologous by as little as 25% sequence identity between the two complete mature proteins. (See generally, Doolittle, R.F., OF URFS and ORFS, University Science Books, CA, 1986.)

A genomic or other appropriate library prepared from the candidate plant source of interest may be probed with conserved sequences from the plant kappa hydroxylase to identify homologously related sequences. Use of an entire cDNA or other sequence may be employed if shorter probe sequences are not identified. Positive clones are then analyzed by restriction enzyme digestion and/or sequencing. When a genomic library is used, one or more sequences may be identified providing both the coding region, as well as the transcriptional regulatory elements of the kappa hydroxylase gene from such plant source. Probes can also be considerably shorter than the

entire sequence. Oligonucleotides may be used, for example, but should be at least about 10, preferably at least about 15, more preferably at least 20 nucleotides in length. When shorter
5 length regions are used for comparison, a higher degree of sequence identity is required than for longer sequences. Shorter probes are often particularly useful for polymerase chain reactions (PCR), especially when highly conserved sequences
10 can be identified (See Gould, et al., 1989 for examples of the use of PCR to isolate homologous genes from taxonomically diverse species).

When longer nucleic acid fragments are employed (>100 bp) as probes, especially when
15 using complete or large cDNA sequences, one would screen with low stringencies (for example, 40-50°C below the melting temperature of the probe) in order to obtain signal from the target sample with 20-50% deviation, i.e., homologous sequences.
20 (Beltz, et al. 1983).

In a preferred embodiment, a plant kappa hydroxylase of this invention will have at least
60% overall amino acid sequence similarity with the exemplified plant kappa hydroxylase. In
25 particular, kappa hydroxylases which are obtainable from an amino acid or nucleic acid sequence of a castor or lesquerella kappa hydroxylase are especially preferred. The plant

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kappa hydroxylases may have preferential activity toward longer or shorter chain fatty acyl substrates. Plant fatty acyl hydroxylases having oleate-12-hydroxylase activity and eicosenoate-14-hydroxylase activity are both considered homologously related proteins because of in vitro evidence (Howling et al., 1972), and evidence disclosed herein, that the castor kappa hydroxylase will act on both substrates.

Hydroxylated fatty acids may be subject to further enzymatic modification by other enzymes which are normally present or are introduced by genetic engineering methods. For example, 14-hydroxy-11,17-eicosadienoic acid, which is present in some *Lesquerella* species (Smith 1985), is thought to be produced by desaturation of 14-hydroxy-11-eicosenoic acid.

Again, not only can gene clones and materials derived thereof be used to identify homologous plant fatty acyl hydroxylases, but the resulting sequences obtained therefrom may also provide a further method to obtain plant fatty acyl hydroxylases from other plant sources. In particular, PCR may be a useful technique to obtain related plant fatty acyl hydroxylases from sequence data provided herein. One skilled in the art will be able to design oligonucleotide probes based upon sequence comparisons or regions of

typically highly conserved sequence. Of special interest are polymerase chain reaction primers based on the conserved regions of amino acid sequence between the castor kappa hydroxylase and the *L. fendleri* hydroxylase (SEQ ID NO:4).

Details relating to the design and methods for a PCR reaction using these probes are described more fully in the examples.

It should also be noted that the fatty acyl hydroxylases of a variety of sources can be used to investigate fatty acid hydroxylation events in a wide variety of plant and *in vivo* applications. Because all plants synthesize fatty acids via a common metabolic pathway, the study and/or application of one plant fatty acid hydroxylase to a heterologous plant host may be readily achieved in a variety of species.

Once the nucleic acid sequence is obtained, the transcription, or transcription and translation (expression), of the plant fatty acyl hydroxylases in a host cell is desired to produce a ready source of the enzyme and/or modify the composition of fatty acids found therein in the form of free fatty acids, esters (particularly esterified to glycerolipids or as components of wax esters), estolides, or ethers. Other useful applications may be found when the host cell is a plant host cell, *in vitro* and *in vivo*. For

example, by increasing the amount of an kappa hydroxylase available to the plant, an increased percentage of ricinoleate or lesqueroleate (14-hydroxy-11-eicosenoic acid) may be provided.

5

Kappa hydroxylase

By this invention, a mechanism for the biosynthesis of ricinoleic acid in plants is demonstrated. Namely, that a specific plant kappa hydroxylase having preferential activity toward fatty acyl substrates is involved in the accumulation of hydroxylated fatty acids in at least some plant species. The use of the terms ricinoleate or ricinoleic acid (or lesqueroleate or lesquerolic acid, densipoleate etc.) is intended to include the free acids, the ACP and CoA esters, the salts of these acids, the glycerolipid esters (particularly the triacylglycerol esters), the wax esters, the estolides and the ether derivatives of these acids.

The determination that plant fatty acyl hydroxylases are active in the *in vivo* production of hydroxylated fatty acids suggests several possibilities for plant enzyme sources. In fact, hydroxylated fatty acids are found in some natural plant species in abundance. For example, three

hydroxy fatty acids related to ricinoleate occur
in major amounts in seed oils from various
Lesquerella species. Of particular interest,
lesquerolic acid is a 20 carbon homolog of
5 ricinoleate with two additional carbons at the
carboxyl end of the chain (Smith 1985). Other
natural plant sources of hydroxylated fatty acids
include but are not limited to seeds of the Linum
genus, seeds of Wrightia species, Lycopodium
10 species, Strophanthus species, Convolvulaceae
species, Calendula species and many others (van de
Loo et al., 1993).

Plants having significant presence of
ricinoleate or lesqueroleate or desaturated other
15 or modified derivatives of these fatty acids are
preferred candidates to obtain naturally-derived
kappa hydroxylases. For example, *Lesquerella*
densipila contains a diunsaturated 18 carbon fatty
acid with a hydroxyl group (van de Loo et al.,
20 1993) that is thought to be produced by an enzyme
that is closely related to the castor kappa
hydroxylase, according to the theory on which this
invention is based. In addition, a comparison
between kappa hydroxylases and between plant fatty
25 acyl hydroxylases which introduce hydroxyl groups
at positions other than the 12-carbon of oleate or
the 14-carbon of lesqueroleate or on substrates
other than oleic acid and icosanoic acid may yield

insights for protein modeling or other
modifications to create synthetic hydroxylases as
discussed above. For example, on the basis of
information gained from structural comparisons of
5 the $\Delta 12$ desaturases and the kappa hydroxylase, we
envision making genetic modifications in the
structural genes for $\Delta 12$ desaturases that convert
these desaturases to kappa-hydroxylases. We also
envision making changes in $\Delta 15$ hydroxylases that
10 convert these to hydroxylases with comparable
substrate specificity to the desaturases (e.g.,
conversion of $18:2^{\Delta 9,12}$ to $15OH-18:2^{\Delta 9,12}$. Since
the difference between a hydroxylase and a
desaturases concerns the disposition of one
15 proton, we envision that by systematically
changing the charged groups in the region of the
enzyme near the active site, we can effect this
change.

Especially of interest are fatty acyl
20 hydroxylases which demonstrate activity toward
fatty acyl substrates other than oleate, or which
introduce the hydroxyl group at a location other
than the C12 carbon. As described above, other
plant sources may also provide sources for these
25 enzymes through the use of protein purification,
nucleic acid probes, antibody preparations,
protein modeling, or sequence comparisons, for
example, and of special interest are the

respective amino acid and nucleic acid sequences
corresponding to such plant fatty acyl
hydroxylases. Also as previously described, once
a nucleic acid sequence is obtained for the given
5 plant hydroxylase, further plant sequences may be
compared and/or probed to obtain homologously
related DNA sequences thereto and so on.

Genetic Engineering Applications

10 As is well known in the art, once a cDNA
clone encoding a plant kappa hydroxylase is
obtained, it may be used to obtain its
corresponding genomic nucleic acid sequences
15 thereto.

The nucleic acid sequences which encode plant
kappa hydroxylases may be used in various
constructs, for example, as probes to obtain
further sequences from the same or other species.
20 Alternatively, these sequences may be used in
conjunction with appropriate regulatory sequences
to increase levels of the respective hydroxylase
of interest in a host cell for the production of
hydroxylated fatty acids or study of the enzyme *in*
25 *vitro* or *in vivo* or to decrease or increase levels
of the respective hydroxylase of interest for some
applications when the host cell is a plant entity,
including plant cells, plant parts (including but

not limited to seeds, cuttings or tissues) and plants.

A nucleic acid sequence encoding a plant kappa hydroxylase of this invention may include genomic, cDNA or mRNA sequence. By "encoding" is meant that the sequence corresponds to a particular amino acid sequence either in a sense or anti-sense orientation. By "recombinant" is meant that the sequence contains a genetically engineered modification through manipulation via mutagenesis, restriction enzymes, and the like. A cDNA sequence may or may not encode pre-processing sequences, such as transit or signal peptide sequences. Transit or signal peptide sequences facilitate the delivery of the protein to a given organelle and are frequently cleaved from the polypeptide upon entry into the organelle, releasing the "mature" sequence. The use of the precursor DNA sequence is preferred in plant cell expression cassettes.

Furthermore, as discussed above the complete genomic sequence of the plant kappa hydroxylase may be obtained by the screening of a genomic library with a probe, such as a cDNA probe, and isolating those sequences which regulate expression in seed tissue. In this manner, the transcription and translation initiation regions, introns, and/or transcript termination regions of

the plant kappa hydroxylase may be obtained for use in a variety of DNA constructs, with or without the kappa hydroxylase structural gene. Thus, nucleic acid sequences corresponding to the plant kappa hydroxylase of this invention may also provide signal sequences useful to direct transport into an organelle 5' upstream non-coding regulatory regions (promoters) having useful tissue and timing profiles, 3' downstream non-coding regulatory region useful as transcriptional and translational regulatory regions and may lend insight into other features of the gene.

Once the desired plant kappa hydroxylase nucleic acid sequence is obtained, it may be manipulated in a variety of ways. Where the sequence involves non-coding flanking regions, the flanking regions may be subjected to resection, mutagenesis, etc. Thus, transitions, transversions, deletions, and insertions may be performed on the naturally occurring sequence. In addition, all or part of the sequence may be synthesized. In the structural gene, one or more codons may be modified to provide for a modified amino acid sequence, or one or more codon mutations may be introduced to provide for a convenient restriction site or other purpose involved with construction or expression. The structural gene may be further modified by

employing synthetic adapters, linkers to introduce one or more convenient restriction sites, or the like.

5 The nucleic acid or amino acid sequences encoding a plant kappa hydroxylase of this invention may be combined with other non-native, or "heterologous", sequences in a variety of ways. By "heterologous" sequences is meant any sequence which is not naturally found joined to the plant
10 kappa hydroxylase, including, for example, combination of nucleic acid sequences from the same plant which are not naturally found joined together.

15 The DNA sequence encoding a plant kappa hydroxylase of this invention may be employed in conjunction with all or part of the gene sequences normally associated with the kappa hydroxylase. In its component parts, a DNA sequence encoding kappa hydroxylase is combined in a DNA construct
20 having, in the 5' to 3' direction of transcription, a transcription initiation control region capable of promoting transcription and translation in a host cell, the DNA sequence encoding plant kappa hydroxylase and a
25 transcription and translation termination region.

Potential host cells include both prokaryotic and eukaryotic cells. A host cell may be unicellular or found in a multicellular

differentiated or undifferentiated organism
depending upon the intended use. Cells of this
invention may be distinguished by having a plant
kappa hydroxylase foreign to the wild-type cell
5 present therein, for example, by having a
recombinant nucleic acid construct encoding a
plant kappa hydroxylase therein.

Depending upon the host, the regulatory
regions will vary, including regions from viral,
10 plasmid or chromosomal genes, or the like. For
expression in prokaryotic or eukaryotic
microorganisms, particularly unicellular hosts, a
wide variety of constitutive or regulatable
promoters may be employed. Expression in a
15 microorganism can provide a ready source of the
plant enzyme. Among transcriptional initiation
regions which have been described are regions from
bacterial and yeast hosts, such as *E. coli*, *B.*
subtilis, *Saccharomyces cerevisiae*, including
20 genes such as beta-galactosidase, T7 polymerase,
tryptophan E and the like.

For the most part, the constructs will
involve regulatory regions functional in plants
which provide for modified production of plant
25 kappa hydroxylase with resulting modification of
the fatty acid composition. The open reading
frame, coding for the plant kappa hydroxylase or
functional fragment thereof will be joined at its

5' end to a transcription initiation regulatory region such as the wild-type sequence naturally found 5' upstream to the kappa hydroxylase structural gene. Numerous other transcription initiation regions are available which provide for a wide variety of constitutive or regulatable, e.g., inducible, transcription of the structural gene functions.

Among transcriptional initiation regions used for plants are such regions associated with the structural genes such as for nopaline and mannopine synthases, or with napin, soybean β -conglycinin, oleosin, 12S storage protein, the cauliflower mosaic virus 35S promoters and the like. The transcription/ translation initiation regions corresponding to such structural genes are found immediately 5' upstream to the respective start codons.

In embodiments wherein the expression of the kappa hydroxylase protein is desired in a plant host, the use of all or part of the complete plant kappa hydroxylase gene is desired; namely all or part of the 5' upstream non-coding regions (promoter) together with the structural gene sequence and 3' downstream non-coding regions may be employed. If a different promoter is desired, such as a promoter native to the plant host of interest or a modified promoter, i.e., having

transcription initiation regions derived from one
gene source and translation initiation regions
derived from a different gene source, including
the sequence encoding the plant kappa hydroxylase
of interest, or enhanced promoters, such as double
35S CaMV promoters, the sequences may be joined
together using standard techniques.

For such applications when 5' upstream non-
coding regions are obtained from other genes
regulated during seed maturation, those
preferentially expressed in plant embryo tissue,
such as transcription initiation control regions
from the *B. napus* napin gene, or the Arabidopsis
12S storage protein, or soybean β -conglycinin
(Bray et al., 1987), or the *L. fendleri* kappa
hydroxylase promoter described herein are desired.
Transcription initiation regions which are
preferentially expressed in seed tissue, i.e.,
which are undetectable in other plant parts, are
considered desirable for fatty acid modifications
in order to minimize any disruptive or adverse
effects of the gene product.

Regulatory transcript termination regions may
be provided in DNA constructs of this invention as
well. Transcript termination regions may be
provided by the DNA sequence encoding the plant
kappa hydroxylase or a convenient transcription
termination region derived from a different gene

source, for example, the transcript termination region which is naturally associated with the transcript initiation region. Where the transcript termination region is from a different gene source, it will contain at least about 0.5 kb, preferably about 1-3 kb of sequence 3' to the structural gene from which the termination region is derived.

Plant expression or transcription constructs having a plant kappa hydroxylase as the DNA sequence of interest for increased or decreased expression thereof may be employed with a wide variety of plant life, particularly, plant life involved in the production of vegetable oils for edible and industrial uses. Most especially preferred are temperate oilseed crops. Plants of interest include, but are not limited to rapeseed (Canola and high erucic acid varieties), Crambe, *Brassica juncea*, *Brassica nigra*, meadowfoam, flax, sunflower, safflower, cotton, *Cuphea*, soybean, peanut, coconut and oil palms and corn. An important criterion in the selection of suitable plants for the introduction on the kappa hydroxylase is the presence in the host plant of a suitable substrate for the hydroxylase. Thus, for example, production of ricinoleic acid will be best accomplished in plants that normally have high levels of oleic acid in seed lipids.

Similarly, production of lesquerolic acid will best be accomplished in plants that have high levels of icosenoic acid in seed lipids.

Depending on the method for introducing the recombinant constructs into the host cell, other DNA sequences may be required. Importantly, this invention is applicable to dicotyledons and monocotyledons species alike and will be readily applicable to new and/or improved transformation and regulation techniques. The method of transformation is not critical to the current invention; various methods of plant transformation are currently available. As newer methods are available to transform crops, they may be directly applied hereunder. For example, many plant species naturally susceptible to *Agrobacterium* infection may be successfully transformed via tripartite or binary vector methods of *Agrobacterium* mediated transformation. In addition, techniques of microinjection, DNA particle bombardment, electroporation have been developed which allow for the transformation of various monocot and dicot plant species.

In developing the DNA construct, the various components of the construct or fragments thereof will normally be inserted into a convenient cloning vector which is capable of replication in a bacterial host, e.g., *E. coli*. Numerous vectors

exist that have been described in the literature.
After each cloning, the plasmid may be isolated
and subjected to further manipulation, such as
restriction, insertion of new fragments, ligation,
5 deletion, insertion, resection, etc., so as to
tailor the components of the desired sequence.
Once the construct has been completed, it may then
be transferred to an appropriate vector for
further manipulation in accordance with the manner
10 of transformation of the host cell.

Normally, included with the DNA construct
will be a structural gene having the necessary
regulatory regions for expression in a host and
providing for selection of transformant cells.

15 The gene may provide for resistance to a cytotoxic
agent, e.g., antibiotic, heavy metal, toxin, etc.,
complementation providing prototrophy to an
auxotrophic host, viral immunity or the like.
Depending upon the number of different host
20 species the expression construct or components
thereof are introduced, one or more markers may be
employed, where different conditions for selection
are used for the different hosts.

It is noted that the degeneracy of the DNA
25 code provides that some codon substitutions are
permissible of DNA sequences without any
corresponding modification of the amino acid
sequence.

As mentioned above, the manner in which the DNA construct is introduced into the plant host is not critical to this invention. Any method which provides for efficient transformation may be employed. Various methods for plant cell transformation include the use of Ti- or Ri-plasmids, microinjection, electroporation, infiltration, imbibition, DNA particle bombardment, liposome fusion, DNA bombardment or the like. In many instances, it will be desirable to have the construct bordered on one or both sides of the T-DNA, particularly having the left and right borders, more particularly the right border. This is particularly useful when the construct uses *A. tumefaciens* or *A. rhizogenes* as a mode for transformation, although the T-DNA borders may find use with other modes of transformation.

Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the *Agrobacterium* host for homologous recombination with T-DNA or the Ti- or Ri-plasmid present in the *Agrobacterium* host. The Ti- or Ri-plasmid containing the T-DNA for recombination may be armed (capable of causing gall formation) or disarmed (incapable of causing gall), the latter being permissible, so long as the *vir* genes are present in the transformed

Agrobacterium host. The armed plasmid can give a mixture of normal plant cells and gall.

In some instances where *Agrobacterium* is used as the vehicle for transforming plant cells, the expression construct bordered by the T-DNA border(s) will be inserted into a broad host spectrum vector, there being broad host spectrum vectors described in the literature. Commonly used is pRK2 or derivatives thereof. See, for example, Ditta et al., (1980), which is incorporated herein by reference. Included with the expression construct and the T-DNA will be one or more markers, which allow for selection of transformed *Agrobacterium* and transformed plant cells. A number of markers have been developed for use with plant cells, such as resistance to kanamycin, the aminoglycoside G418, hygromycin, or the like. The particular marker employed is not essential to this invention, one or another marker being preferred depending on the particular host and the manner of construction.

For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time for transformation, the bacteria killed, and the plant cells cultured in an appropriate selective medium. Once callus forms, shoot formation can be encouraged by employing the

appropriate plant hormones in accordance with
known methods and the shoots transferred to
rooting medium for regeneration of plants. The
plants may then be grown to seed and the seed used
5 to establish repetitive generations and for
isolation of vegetable oils.

Using Hydroxylase Genes to Alter the Activity of Fatty Acid Desaturases

10 A widely acknowledged goal of current efforts
to improve the nutritional quality of edible plant
oils, or to facilitate industrial applications of
plant oils, is to alter the level of desaturation
of plant storage lipids (Topfer et al., 1995). In
15 particular, in many crop species it is considered
desirable to reduce the level of polyunsaturation
of storage lipids and to increase the level of
oleic acid. The precise amount of the various
fatty acids in a particular plant oil varies with
20 the intended application. Thus, it is desirable
to have a robust method that will permit genetic
manipulation of the level of unsaturation to any
desired level.

Substantial progress has recently been made
25 in the isolation of genes encoding plant fatty
acid desaturases (reviewed in Topfer et al.,
1995). These genes have been introduced into
various plant species and used to alter the level

of fatty acid unsaturation in one of three ways.

First, the genes can be placed under transcriptional control of a strong promoter so that the amount of the corresponding enzyme is increased. In some cases this leads to an increase in the amount of the fatty acid that is the product of the reaction catalyzed by the enzyme. For example, Arondel et al. (1992) increased the amount of linolenic acid (18:3) in tissues of transgenic Arabidopsis plants by placing the endoplasmic reticulum-localized fad3 gene under transcriptional control of the strong constitutive cauliflower mosaic virus 35S promoter.

A second method of using cloned genes to alter the level of fatty acid unsaturation is to cause transcription of all or part of a gene in transgenic tissues so that the transcripts have an antisense orientation relative to the normal mode of transcription. This has been used by a number of laboratories to reduce the level of expression of one or more desaturase genes that have significant nucleotide sequence homology to the gene used in the construction of the antisense gene (reviewed in Topfer et al.). For instance, antisense repression of the oleate $\Delta 12$ -desaturase in transgenic rapeseed resulted in a strong increase in oleic acid content (cf., Topfer et

al., 1995).

A third method for using cloned genes to alter fatty acid desaturation is to exploit the phenomenon of cosuppression or "gene-silencing" (Matzke et al., 1995). Although the mechanisms responsible for gene silencing are not known in any detail, it has frequently been observed that in transgenic plants, expression of an introduced gene leads to inactivation of homologous endogenous genes.

For example, high-level sense expression of the Arabidopsis fad8 gene, which encodes a chloroplast-localized $\Delta 15$ -desaturase, in transgenic Arabidopsis plants caused suppression of the endogenous copy of the fad8 gene and the homologous fad7 gene (which encodes an isozyme of the fad8 gene) (Gibson et al., 1994). The fad7 and fad8 genes are only 76% identical at the nucleotide level. At the time of publication, this example represented the most divergent pair of plant genes for which cosuppression had been observed.

In view of previous evidence concerning the relatively high level of nucleotide sequence homology required to obtain cosuppression, it is not obvious to one skilled in the art that sense expression in transgenic plants of the castor fatty acyl hydroxylase of this invention would

significantly alter the amount of unsaturation of storage lipids.

However, we have established that fatty acyl hydroxylase genes can be used for this purpose as taught in Example 4 of this invention. Of particular importance, this invention teaches the use of fatty acyl hydroxylase genes to increase the proportion of oleic acid in transgenic plant tissues. The mechanism by which expression of the gene exerts this effect is not known but may be due to one of several possibilities which are elaborated upon in Example 4.

The invention now being generally described, it will be more readily understood by reference to the following examples which are included for purposes of illustration only and are not intended to limit the present invention.

EXAMPLES

In the experimental disclosure which follows, all temperatures are given in degrees centigrade ($^{\circ}$), weights are given in grams (g), milligram (mg) or micrograms (μ g), concentrations are given as molar (M), millimolar (mM) or micromolar (μ M) and all volumes are given in liters (l), microliters (μ l) or milliliters (ml), unless otherwise indicated.

EXAMPLE 1 - PRODUCTION OF NOVEL HYDROXYLATED FATTY
ACIDS IN ARABIDOPSIS THALIANA

Overview

5 The kappa hydroxylase encoded by the
previously described fah12 gene from Castor (U.S.
Patent application 08/320,982) was used to produce
ricinoleic acid, lesquerolic acid, densipolic acid
and auricolic acid in transgenic Arabidopsis
plants. This example specifically discloses the
10 method taught in Example 2 of U.S. Patent
application 08/320,982.

Production of transgenic plants

15 A variety of methods have been developed to
insert a DNA sequence of interest into the genome
of a plant host to obtain the transcription and
translation of the sequence to effect phenotypic
changes. The following methods represent only one
of many equivalent means of producing transgenic
20 plants and causing expression of the hydroxylase
gene.

Arabidopsis plants were transformed, by
Agrobacterium-mediated transformation, with the
kappa hydroxylase encoded by the Castor fah12 gene
25 on binary Ti plasmid pB6. This plasmid was
previously used to transform *Nicotiana tabacum* for
the production of ricinoleic acid (U.S. Patent
application 08/320,982).

Inoculums of *Agrobacterium tumefaciens* strain GV3101 containing binary Ti plasmid pB6 were plated on L-broth plates containing 50 µg/ml kanamycin and incubated for 2 days at 30°C.

5 Single colonies were used to inoculate large liquid cultures (L-broth medium with 50 mg/l rifampicin, 110 mg/l gentamycin and 200 mg/l kanamycin) to be used for the transformation of *Arabidopsis* plants.

10 *Arabidopsis* plants were transformed by the *in planta* transformation procedure essentially as described by Bechtold et al., (1993). Cells of *A. tumefaciens* GV3101(pB6) were harvested from liquid cultures by centrifugation, then resuspended in
15 infiltration medium at OD₆₀₀ = 0.8 (Infiltration medium was Murashige and Skoog macro and micronutrient medium (Sigma Chemical Co., St. Louis, MO) containing 10 mg/l 6-benzylaminopurine and 5% glucose). Batches of 12-15 plants were
20 grown for 3 to 4 weeks in natural light at a mean daily temperature of approximately 25°C in 3.5 inch pots containing soil. The intact plants were immersed in the bacterial suspension then transferred to a vacuum chamber and placed under
25 600 mm of vacuum produced by a laboratory vacuum pump until tissues appeared uniformly water-soaked (approximately 10 min). The plants were grown at 25°C under continuous light (100 µmol m⁻² s⁻¹

irradiation in the 400 to 700 nm range) for four weeks. The seeds obtained from all the plants in a pot were harvested as one batch. The seeds were sterilized by sequential treatment for 2 min with ethanol followed by 10 min in a mixture of household bleach (Chlorox), water and Tween-80 (50%, 50%, 0.05%) then rinsed thoroughly with sterile water. The seeds were plated at high density (2000 to 4000 per plate) onto agar-solidified medium in 100 mm petri plates containing 1/2 X Murashige and Skoog salts medium enriched with B5 vitamins (Sigma Chemical Co., St. Louis, MO) and containing kanamycin at 50 mg/l. After incubation for 48 h at 4°C to stimulate germination, seedlings were grown for a period of seven days until transformants were clearly identifiable as healthy green seedlings against a background of chlorotic kanamycin-sensitive seedlings. The transformants were transferred to soil for two weeks before leaf tissue could be used for DNA and lipid analysis. More than 20 transformants were obtained.

DNA was extracted from young leaves from transformants to verify the presence of an intact fah12 gene. The presence of the transgene in a number of the putative transgenic lines was verified by using the polymerase chain reaction to amplify the insert from pB6. The primers used

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were HF2 = GCTCTTTTGTGCGCTCATTC (SEQ ID NO:12) and
HR1 = CGGTACCAGAAAACGCCTTG (SEQ ID NO:13), which
were designed to allow the amplification of a 700
bp fragment. Approximately 100 ng of genomic DNA
5 was added to a solution containing 25 pmol of each
primer, 1.5 U Taq polymerase (Boehringer Mannheim),
200 uM of dNTPs, 50 mM KCl, 10 mM Tris.Cl (pH 9),
0.1% (v/v) Triton X-100, 1.5 mM MgCl₂, 3% (v/v)
formamide, to a final volume of 50 µl.
10 Amplifications conditions were: 4 min denaturation
step at 94°C, followed by 30 cycles of 92°C for 1
min, 55°C for 1 min, 72°C for 2 min. A final
extension step closed the program at 72°C for 5
min. Transformants could be positively identified
15 after visualization of a characteristic 1 kb
amplified fragment on an ethidium bromide stained
agarose gel. All transgenic lines tested gave a
PCR product of a size consistent with the expected
genotype, confirming that the lines were, indeed,
20 transgenic. All further experiments were done
with three representative transgenic lines of the
wild type designated as 1-3, 4D, 7-4 and one
transgenic line of the fad2 mutant line JB12. The
transgenic JB12 line was included in order to test
25 whether the increased accumulation of oleic acid
in this mutant would have an effect on the amount
of ricinoleic acid that accumulated in the
transgenic plants.

Analysis of transgenic plants

Leaves and seeds from fah12 transgenic Arabidopsis plants were analyzed for the presence of hydroxylated fatty acids using gas chromatography. Lipids were extracted from 100-200 mg leaf tissue or 50 seeds. Fatty acid methyl esters (FAMES) were prepared by placing tissue in 1.5 ml of 1.0 M methanolic HCl (Supelco Co.) in a 13 x 100 mm glass screw-cap tube capped with a teflon-lined cap and heated to 80°C for 2 hours. Upon cooling, 1 ml petroleum ether was added and the FAMES removed by aspirating off the ether phase which was then dried under a nitrogen stream in a glass tube. One hundred μ l of N,O-bis(Trimethylsilyl) trifluoroacetamide (BSTFA; Pierce Chemical Co) and 200 μ l acetonitrile was added to derivatize the hydroxyl groups. The reaction was carried out at 70°C for 15 min. The products were dried under nitrogen, redissolved in 100 μ l chloroform and transferred to a gas chromatograph vial. Two μ l of each sample were analyzed on a SP2340 fused silica capillary column (30 m, 0.75 mm ID, 0.20 mm film, Supelco), using a Hewlett-Packard 5890 II series Gas Chromatograph. The samples were not split, the temperature program was 195°C for 18 min, increased to 230°C at 25°C/min, held at 230°C for 5 min then down to 195°C at 25°C/min., and flame ionization detectors

were used.

The chromatographic elution time of methyl esters and O-TMS derivatives of ricinoleic acid, lesquerolic acid and auricolic acid was

established by GC-MS of lipid samples from seeds of *L. fendleri* and comparison to published chromatograms of fatty acids from this species (Carlson et al., 1990). A O-

TMS-methyl-ricinoleate standard was prepared from ricinoleic acid obtained from Sigma Chemical Co (St, Louis, MO). O-TMS-methyl-lesqueroleate and O-TMS-methyl-auricoleate standards were prepared from triacylglycerols purified from seeds of *L. fendleri*. The mass spectrum of O-

TMS-methyl-ricinoleate, O-TMS-methyl-densipoleate, O-TMS-methyl-lesqueroleate, and O-TMS-methyl-auricoleate are shown in Figures 1A-D, respectively. The structures of the characteristic ions produced during mass spectrometry of these derivatives are shown in Figure 2.

Lipid extracted from transgenic tissues were analyzed by gas chromatography and mass spectrometry for the presence of hydroxylated fatty acids. As a matter of reference, the average fatty acid composition of leaves in *Arabidopsis* wild type and *fad2* mutant lines was reported by Miquel and Browse (1992). Gas

chromatograms of methylated and silylated fatty acids from seeds of wild type and a fah12 transgenic wild type plant are shown in Figures 3A and 3B, respectively. The profiles are very similar except for the presence of three small but distinct peaks at 14.3, 15.9 and 18.9 minutes. A very small peak at 20.15 min was also evident. The elution time of the peaks at 14.3 and 18.9 min corresponded precisely to that of comparably prepared ricinoleic and lesquerolic standards, respectively. No significant differences were observed in lipid extracts from leaves or roots of the wild type and the fah12 transgenic wild type lines (Table 1). Thus, in spite of the fact that the fah12 gene is expressed throughout the plant, we observed effects on fatty acid composition only in seed tissue. A similar observation was described previously for transgenic fah12 tobacco in patent application No. 08/320,982.

Table 1. Fatty acid composition of lipids from transgenic and wild type Arabidopsis. The values are the means obtained from analysis of samples from three independent transgenic lines, or three independent samples of wild type and fad2 lines.

Fatty acid	Seed				Leaf		Root	
	WT	FAH12/ WT	FAH12/f ad2	JB12	WT	FAH12/W T	WT	FAH12/W T
16:0	8.5	8.2	6.4	6.1	16.5	17.5	23.9	24.9
16:3	0	0	0	0	10.1	9.8	0	0
18:0	3.2	3.5	2.9	3.5	1.3	1.2	2.0	1.9
18:1	15.4	26.3	43.4	47.8	2.4	3.4	5.4	3.2
18:2	27.0	21.4	10.2	7.2	15.1	14.0	32.2	29.4
18:3	22.0	16.6	-	9.7	36.7	36.0	26.7	30.6
20:1	14.0	14.3	-	13.1	0	0	0	0
22:1	2.0	1.0	0.5	0.5	0	0	0	0
24:1	2.5	1.7	2.0	1.6	0	0	0	0
18:1-OH	0	0.4	0.3	0	0	0	0	0
18:2-OH	0	0.4	0.3	0	0	0	0	0
20:1-OH	0	0.2	0.1	0	0	0	0	0
20:2-OH	0	0.1	0.1	0	0	0	0	0

In order to confirm that the observed new peaks in the transgenic lines corresponded to derivatives of ricinoleic, lesquerolic, densipolic and auricolic acids, mass spectrometry was used.

5 The fatty acid derivatives were resolved by gas chromatography as described above except that a Hewlett-Packard 5971 series mass selective detector was used in place of the flame ionization detector used in the previous experiment. The
10 spectra of the four new peaks in Figure 3B (peak numbers 10, 11, 12 and 13) are shown in Figures 4A-D, respectively. Comparison of the spectrum obtained for the standards with that obtained for the four peaks from the transgenic lines confirms
15 the identity of the four new peaks. On the basis of the three characteristic peaks at M/Z 187, 270 and 299, peak 10 is unambiguously identified as O-TMS-methylricinoleate. On the basis of the three characteristic peaks at M/Z 185, 270 and 299, peak
20 11 is unambiguously identified as O-TMS-methyldensipoleate. On the basis of the three characteristic peaks at M/Z 187, 298 and 327, peak 12 is unambiguously identified as O-TS-
25 methyllesqueroleate. On the basis of the three characteristic peaks at M/Z 185, 298 and 327, peak 13 is unambiguously identified as O-TMS-methylauricoleate.

These results unequivocally demonstrate the

identity of the fah12 cDNA as encoding a
hydroxylase that hydroxylates both oleic acid to
produce ricinoleic acid and also hydroxylates
icosenoic acid to produce lesquerolic acid. These
5 results also provide additional evidence that the
hydroxylase can be functionally expressed in a
heterologous plant species in such a way that the
enzyme is catalytically functional. These results
also demonstrate that expression of this
10 hydroxylase gene leads to accumulation of
ricinoleic, lesquerolic, densipolic and auricolic
acids in a plant species that does not normally
accumulate hydroxylated fatty acids in extractable
lipids.

15 The presence of lesquerolic acid in the
transgenic plants was anticipated in the previous
patent application (No. 08/320,982) based on the
biochemical evidence suggesting broad substrate
specificity of the kappa hydroxylase. By
20 contrast, the accumulation of densipolic and
auricolic acids was less predictable. Since
Arabidopsis does not normally contain significant
quantities of the non-hydroxylated precursors of
these fatty acids which could serve as substrates
25 for the hydroxylase, it appears that one or more
of the three n-3 fatty acid desaturases known in
Arabidopsis (eg., fad3, fad7, fad8; reviewed in
Gibson et al., 1995) are capable of desaturating

the hydroxylated compounds at the n-3 position.
That is, densipolic acid is produced by the action
of an n-3 desaturase on ricinoleic acid.

Auricollic acid is produced by the action of an n-3
5 desaturase on lesquerolic acid. Because it is
located in the endoplasmic reticulum, the fad3
desaturase is almost certainly responsible. This
can be tested in the future by producing fah12-
containing transgenic plants of the fad3-deficient
10 mutant of Arabidopsis (similar experiments can be
done with fad7 and fad8). It is also formally
possible that the enzymes that normally elongate
18:1^{cis} Δ^9 to 20:1^{cis} Δ^{11} may elongate 12OH-
18:1^{cis} Δ^9 to 14OH-20:1^{cis} Δ^{11} , and 12OH-
15 18:2^{cis} $\Delta^{9,15}$ to 14OH-20:2^{cis} $\Delta^{11,17}$.

The amount of the various fatty acids in
seed, leaf and root lipids of the control and
transgenic plants is also presented in Table 1.
Although the amount of hydroxylated fatty acids
20 produced in this example is less than desired for
commercial production of ricinoleate and other
hydroxylated fatty acids from plants, we envision
numerous improvements of this invention that will
increase the level of accumulation of hydroxylated
25 fatty acids in plants that express the fah12 or
related hydroxylase genes. Improvements in the
level and tissue specificity of expression of the
hydroxylase gene is envisioned. Methods to

accomplish this by the use of strong, seed-specific promoters such as the *B. napus* napin promoter or the native promoters of the castor fah12 gene or the corresponding hydroxylase gene from *L. fendleri* will be obvious to one skilled in the art. Additional improvements are envisioned to involve modification of the enzymes which cleave hydroxylated fatty acids from phosphatidylcholine, reduction in the activities of enzymes which degrade hydroxylated fatty acids and replacement of acyltransferases which transfer hydroxylated fatty acids to the sn-1, sn-2 and sn-3 positions of glycerolipids. Although genes for these enzymes have not been described in the scientific literature, their utility in improving the level of production of hydroxylated fatty acids can be readily envisioned based on the results of biochemical investigations of ricinoleate synthesis.

Although *Arabidopsis* is not an economically important plant species, it is widely accepted by plant biologists as a model for higher plants. Therefore, the inclusion of this example is intended to demonstrate the general utility of the invention described here and in the previous application (No. 08/320,982) to the modification of oil composition in higher plants. One advantage of studying the expression of this novel

gene in Arabidopsis is the existence in this system of a large body of knowledge on lipid metabolism, as well as the availability of a collection of mutants which can be used to provide
5 useful information on the biochemistry of fatty acid hydroxylation in plant species. Another advantage is the ease of transposing any of the information obtained on metabolism of ricinoleate in Arabidopsis to closely related species such as
10 the crop plants *Brassica napus*, *Brassica juncea* or *Crambe abyssinica* in order to mass produce ricinoleate, lesqueroleate or other hydroxylated fatty acids for industrial use. The kappa hydroxylase is useful for the production of
15 ricinoleate or lesqueroleate in any plant species that accumulates significant levels of the precursors, oleic acid and icosanoic acid. Of particular interest are genetically modified varieties that accumulate high levels of oleic
20 acid. Such varieties are currently available for sunflower and Canola. Production of lesquerolic acid and related hydroxy fatty acids can be achieved in species that accumulate high levels of icosanoic acid or other long chain monoenoic
25 acids. Such plants may in the future be produced by genetic engineering of plants that do not normally make such precursors. Thus, we envision that the use of the kappa hydroxylase is of

general utility.

EXAMPLE 2. ISOLATION OF LESQUERELLA KAPPA
HYDROXYLASE GENOMIC CLONE

5

Overview

Regions of nucleotide sequence that were conserved in both the Castor kappa hydroxylase and the Arabidopsis fad2 Δ 12 fatty acid desaturase were used to design oligonucleotide primers. These were used with genomic DNA from *Lesquerella fendleri* to amplify fragments of several homologous genes. These amplified fragments were then used as hybridization probes to identify full length genomic clones from a genomic library of *L. fendleri*.

Hydroxylated fatty acids are specific to the seed tissue of *Lesquerella* sp., and are not found to any appreciable extent in vegetative tissues. One of the two genes identified by this method was expressed in both leaves and developing seeds and is therefore thought to correspond to the Δ 12 fatty acid desaturase. The other gene was expressed at high levels in developing seeds but was not expressed or was expressed at very low levels in leaves and is the kappa hydroxylase from this species. The identity of the gene as a fatty acyl hydroxylase was established by functional

expression of the gene in yeast.

The identity of this gene will also be established by introducing the gene into transgenic Arabidopsis plants and showing that it causes the accumulation of ricinoleic acid, lesquerolic acid, densipolic acid and auricollic acid in seed lipids. The promoter of this gene is also of utility because it is able to direct expression of a gene specifically in developing seeds at a time when storage lipids are accumulating. This promoter is, therefore, of great utility for many applications in the genetic engineering of seeds, particularly in members of the Brassicacea.

The various steps involved in this process are described in detail below. Unless otherwise indicated, routine methods for manipulating nucleic acids, bacteria and phage were as described by Sambrook et al. (1989).

Isolation of a fragment of the Lesquerella kappa hydroxylase gene

Oligonucleotide primers for the amplification of the *L. fendleri* kappa hydroxylase were designed by choosing regions of high deduced amino acid sequence homology between the Castor kappa hydroxylase and the Arabidopsis $\Delta 12$ desaturase (fad2). Because most amino acids are encoded by

several different codons, these oligonucleotides were designed to encode all possible codons that could encode the corresponding amino acids.

The sequence of these mixed oligonucleotides was:

Oligo 1: TAYWSNCAYMGNMGNCA YCA (SEQ ID NO:14)

Oligo 2: RTGRTGNGCNACRTGNGTRTC (SEQ ID NO:15)

(Where: Y = C+T; W = A+T; S = G+C; N = A+G+C+T; M = A+C; R = A+G)

These oligonucleotides were used to amplify a fragment of DNA from *L. fendleri* genomic DNA by the polymerase chain reaction (PCR) using the following conditions: Approximately 100 ng of genomic DNA was added to a solution containing 25 pmol of each primer, 1.5 U Taq polymerase (Boehringer Mannheim), 200 uM of dNTPs, 50 mM KCl, 10 mM Tris.Cl (pH 9), 0.1% (v/v) Triton X-100, 1.5 mM MgCl₂, 3% (v/v) formamide, to a final volume of 50 µl. Amplifications conditions were: 4 min denaturation step at 94°C, followed by 30 cycles of 92°C for 1 min, 55°C for 1 min, 72°C for 2 min. A final extension step closed the program at 72°C for 5 min.

PCR products of approximately 540 bp were observed following electrophoretic separation of the products of the PCR reaction in agarose gels. Two of these fragments were cloned into pBluescript (Stratagene) to give rise to plasmids

pLesq2 and pLesq3. The sequence of the inserts in these two plasmids was determined by the chain termination method. The sequence of the insert in pLesq2 is presented as Figure 5 (SEQ ID NO:1) and the sequence of the insert in pLesq3 is presented as Figure 6 (SEQ ID NO:2). The high degree of sequence identity between the two clones indicated that they were both potential candidates to be either a $\Delta 12$ desaturase or a gamma hydroxylase.

Northern analysis

In *L. fendleri*, hydroxylated fatty acids are found in large amounts in seed oils but are not found in appreciable amounts in leaves. An important criterion in discriminating between a fatty acyl desaturase and kappa hydroxylase is that the kappa hydroxylase gene is expected to be expressed more highly in tissues which have high level of hydroxylated fatty acids than in other tissues. In contrast, all plant tissues should contain mRNA for an $\omega 6$ fatty acyl desaturase since diunsaturated fatty acids are found in the lipids of all tissues in most or all plants.

Therefore, it was of great interest to determine whether the gene corresponding to pLesq2 was also expressed only in seeds, or is also expressed in other tissues. This question was addressed by testing for hybridization of pLesq2

to RNA purified from developing seeds and from leaves.

Total RNA was purified from developing seeds and young leaves of *L. fendleri* using an Rneasy RNA extraction kit (Qiagen), according to the manufacturer's instructions. RNA concentrations were quantified by UV spectrophotometry at $\lambda=260$ and 280 nm. In order to ensure even loading of the gel to be used for Northern blotting, RNA concentrations were further adjusted after recording fluorescence under UV light of RNA samples stained with ethidium bromide and run on a test denaturing gel.

Total RNA prepared as described above from leaves and developing seeds was electrophoresed through an agarose gel containing formaldehyde (Iba et al., 1993). An equal quantity (10 μ g) of RNA was loaded in both lanes, and RNA standards (0.16-1.77 kb ladder, Gibco-BRL) were loaded in a third lane. Following electrophoresis, RNA was transferred from the gel to a nylon membrane (Hybond N+, Amersham) and fixed to the filter by exposure to UV light.

A 32 P-labelled probe was prepared from insert DNA of clone pLesq2 by random priming and hybridized to the membrane overnight at 52°C, after it had been prehybridized for 2 h. The prehybridization solution contained 5X SSC, 10X

Denhardt's solution, 0.1% SDS, 0.1M KPO₄ pH 6.8,
100 µg/ml salmon sperm DNA. The hybridization
solution had the same basic composition, but no
SDS, and it contained 10% dextran sulfate and 30%
5 formamide. The blot was washed once in 2X SSC,
0.5% SDS at 65°C then in 1X SSC at the same
temperature.

Brief (30 min) exposure of the blot to X-ray
film revealed that the probe pLesq2 hybridized to
10 a single band only in the seed RNA lane (Figure
7). The blot was re-probed with the insert from
pLesq3 gene, which gave bands of similar intensity
in the seed and leaf lanes (Figure 7).

These results show that the gene
15 corresponding to the clone pLesq2 is highly and
specifically expressed in seed of *L. fendleri*. In
conjunction with knowledge of the nucleotide and
deduced amino acid sequence, strong seed-specific
expression of the gene corresponding to the insert
20 in pLesq2 is a convincing indicator of the role of
the enzyme in synthesis of hydroxylated fatty
acids in the seed oil.

25 Characterization of a genomic clone of the gamma hydroxylase

Genomic DNA was prepared from young leaves of
L. fendleri as described by Murray and Thompson
(1980). A *Sau3AI*-partial digest genomic library

constructed in the vector λ DashII (Stratagene,
11011 North Torrey Pines Road, La Jolla CA 92037)
was prepared by partially digesting 500 μ g of DNA,
size-selecting the DNA on a sucrose gradient
5 (Sambrook et al., 1989), and ligating the DNA (12
kb average size) to the *Bam*HI-digested arms of
 λ DashII. The entire ligation was packaged
according to the manufacturer's conditions and
plated on *E. coli* strain XL1-Blue MRA-P2
10 (Stratagene). This yielded 5×10^5 primary
recombinant clones. The library was then
amplified according to the manufacturer's
conditions. A fraction of the genomic library was
plated on *E. coli* XL1-Blue and resulting plaques
15 (150,000) were lifted to charged nylon membranes
(Hybond N+, Amersham), according to the
manufacturer's conditions. DNA was crosslinked to
the filters under UV in a Stratalinker
(Stratagene).

20 Several clones carrying genomic sequences
corresponding to the *L. fenderi* hydroxylase were
isolated by probing the membranes with the insert
from pLesq2 that was PCR-amplified with internal
primers and labelled with 32 P by random priming.
25 The filters were prehybridized for 2 hours at 65°C
in 7% SDS, 1mM EDTA, 0.25 M Na_2HPO_4 (pH 7.2), 1%
BSA and hybridized to the probe for 16 hours in
the same solution. The filters were sequentially

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washed at 65°C in solutions containing 2 X SSC, 1 X SSC, 0.5 X SSC in addition to 0.1 % SDS. A 2.6 kb Xba I fragment containing the complete coding sequence for the gamma-hydroxylase and approximately 1 kb of the 5' upstream region was subcloned into the corresponding site of pBluescript KS to produce plasmid pLesq-Hyd and the sequence determined completely using an automatic sequencer by the dideoxy chain termination method. Sequence data was analyzed using the program DNASIS (Hitachi Company).

The sequence of the insert in clone pLesq-Hyd is shown in Figures 8A-B. The sequence entails 1855 bp of contiguous DNA sequence (SEQ ID NO:3). The clone encodes a 401 bp 5' untranslated region (i.e., nucleotides preceding the first ATG codon), an 1152 bp open reading frame, and a 302 bp 3' untranslated region. The open reading frame encodes a 384 amino acid protein with a predicted molecular weight of 44,370 (SEQ ID NO:4). The amino terminus lacks features of a typical signal peptide (von Heijne, 1985).

The exact translation-initiation methionine has not been experimentally determined, but on the basis of deduced amino acid sequence homology to the Castor kappa hydroxylase (noted below) is thought to be the methionine encoded by the first ATG codon at nucleotide 402.

Comparison of the pLesq-Hyd deduced amino acid sequence with sequences of membrane-bound desaturases and the castor hydroxylase (Figures 9A-B) indicates that pLesq-Hyd is homologous to these genes. This figure shows an alignment of the *L. fendleri* hydroxylase (SEQ ID NO:4) with the castor hydroxylase (van de Loo et al. 1995), the *Arabidopsis fad2* cDNA which encodes an endoplasmic reticulum-localized $\Delta 12$ desaturase (called fad2) (Okuley et al., 1994), two soybean fad2 desaturase clones, a *Brassica napus* fad2 clone, a *Zea mays* fad2 clone and partial sequence of a *R. communis* fad2 clone.

The high degree of sequence homology indicates that the gene products are of similar function. For instance, the overall homology between the Lesquerella hydroxylase and the *Arabidopsis fad2* desaturase was 92.2% similarity and 84.8% identity and the two sequences differed in length by only one amino acid.

Southern hybridization

Southern analysis was used to examine the copy number of the genes in the *L. fendleri* genome corresponding to the clone pLesq-Hyd. Genomic DNA (5 μ g) was digested with *EcoR* I, *Hind* III and *Xba* I and separated on a 0.9% agarose gel. DNA was

alkali-blotted to a charged nylon membrane (Hybond N+, Amersham), according to the manufacturer's protocol. The blot was prehybridized for 2 hours at 65°C in 7% SDS, 1mM EDTA, 0.25 M Na₂HPO₄ (pH 7.2), 1% BSA and hybridized to the probe for 16 hours in the same solution with pLesq-Hyd insert PCR-amplified with internal primers and labelled with ³²P by random priming. The filters were sequentially washed at 65°C in solutions containing 2 X SSC, 1 X SSC, 0.5 X SSC in addition to 0.1 % SDS, then exposed to X-ray film.

The probe hybridized with a single band in each digest of *L. fendleri* DNA (Figure 10), indicating that the gene from which pLesq-Hyd was transcribed is present in a single copy in the *L. fendleri* genome.

Expression of pLesq-Hyd in Transgenic Plants

There are a wide variety of plant promoter sequences which may be used to cause tissue-specific expression of cloned genes in transgenic plants. For instance, the napin promoter and the acyl carrier protein promoters have previously been used in the modification of seed oil composition by expression of an antisense form of a desaturase (Knutson et al. 1992). Similarly, the promoter for the β -subunit of soybean β -conglycinin has been shown to be highly active and

to result in tissue-specific expression in transgenic plants of species other than soybean (Bray et al., 1987). Thus, although we describe the use of the *L. fendleri* kappa hydroxylase promoter in the examples described here, other promoters which lead to seed-specific expression may also be employed for the production of modified seed oil composition. Such modifications of the invention described here will be obvious to one skilled in the art.

Constructs for expression of *L. fendleri* kappa hydroxylase in plant cells are prepared as follows: A 13 kb *SalI* fragment containing the pLesq-Hyg gene was ligated into the *XhoI* site of binary Ti plasmid vector pSLJ44026 (Jones et al., 1992) (Figure 11) to produce plasmid pTi-Hyd and transformed into *Agrobacterium tumefaciens* strains GV3101 by electroporation. Strain GV3101 (Koncz and Schell, 1986) contains a disarmed Ti plasmid. Cells for electroporation were prepared as follows. GV3101 was grown in LB medium with reduced NaCl (5 g/l). A 250 ml culture was grown to $OD_{600} = 0.6$, then centrifuged at 4000 rpm (Sorvall GS-A rotor) for 15 min. The supernatant was aspirated immediately from the loose pellet, which was gently resuspended in 500 ml ice-cold water. The cells were centrifuged as before, resuspended in 30 ml ice-cold water, transferred

to a 30 ml tube and centrifuged at 5000 rpm
(Sorvall SS-34 rotor) for 5 min. This was
repeated three times, resuspending the cells
consecutively in 30 ml ice-cold water, 30 ml ice-
5 cold 10% glycerol, and finally in 0.75 ml ice-cold
10% glycerol. These cells were aliquoted, frozen
in liquid nitrogen, and stored at
-80°C. Electroporations employed a Biorad Gene
pulsar instrument using cold 2 mm-gap cuvettes
10 containing 40 µl cells and 1 µl of DNA in water,
at a voltage of 2.5 KV, and 200 Ohms resistance.
The electroporated cells were diluted with 1 ml
SOC medium (Sambrook et al., 1989, page A2) and
incubated at 28°C for 2-4 h before plating on
15 medium containing kanamycin (50 mg/l).

Arabidopsis thaliana can be transformed with
the *Agrobacterium* cells containing pTi-Hyd as
described in Example 1 above. Similarly, the
presence of hydroxylated fatty acids in the
20 transgeneic *Arabidopsis* plants can be demonstrated
by the methods described in Example 1 above.

Constitutive expression of the *L. fendleri*
hydroxylase in transgenic plants

25 A 1.5 kb *EcoR* I fragment from pLesq-Hyg
comprising the entire coding region of the
hydroxylase was gel purified, then cloned into the
corresponding site of pBluescript KS (Stratagene).

Plasmid DNA from a number of recombinant clones was then restricted with *Pst* I, which should cut only once in the insert and once in the vector polylinker sequence. Release of a 920 bp fragment with *Pst* I indicated the right orientation of the insert for further manipulations. DNA from one such clone was further restricted with *Sal*I, the 5' overhangs filled-in with the Klenow fragment of DNA polymerase I, then cut with *Sac* I. The insert fragment was gel purified, and cloned between the *Sma* I and *Sac* I sites of pBI121 (Clontech) behind the Cauliflower Mosaic Virus 35S promoter. After checking that the sequence of the junction between insert and vector DNA was appropriate, plasmid DNA from a recombinant clone was used to transform *A. tumefaciens* (GV3101). Kanamycin resistant colonies were then used for *in planta* transformation of *A. thaliana* as previously described.

DNA was extracted from kanamycin resistant seedlings and used to PCR-amplify selected fragments from the hydroxylase using nested primers. When fragments of the expected size could be amplified, corresponding plants were grown in the greenhouse or on agar plates, and fatty acids extracted from fully expanded leaves, roots and dry seeds. GC-MS analysis was then performed as previously described to characterize

the different fatty acid species and detect accumulation of hydroxy fatty acids in transgenic tissues.

5 Expression of the *Lesquerella* hydroxylase in yeast

10 In order to demonstrate that the cloned *L. fendleri* gene encoded an oleate-12 hydroxylase, the gene was expressed in yeast cells under transcriptional control of an inducible promoter and the yeast cells were examined for the presence of hydroxylated fatty acids by GC-MS.

15 In a first step, a lambda genomic clone containing the *L. fendleri* hydroxylase gene was cut with *EcoRI*, and a resulting 1400 bp fragment containing the coding sequence of the hydroxylase gene was subcloned in the *EcoRI* site of the pBluescript KS vector (Stratagene). This subclone, pLesqcod, contains the coding region of
20 the *Lesquerella* hydroxylase plus some additional 3' sequence.

25 In a second step, pLesqcod was cut with *HindIII* and *XbaI*, and the insert fragment was cloned into the corresponding sites of the yeast expression vector pYes2 (In Vitrogen; Figure 12). This subclone, pLesqYes, contains the *L. fendleri* hydroxylase in the sense orientation relative to the 3' side of the *Gall* promoter. This promoter

is inducible by the addition of galactose to the growth medium, and is repressed upon addition of glucose. In addition, the vector carries origins of replication allowing the propagation of pLesqYes in both yeast and *E. coli*.

Transformation of *S. cerevisiae* host strain
CGY2557

Yeast strain CGY2557 (*MAT* α , *GAL*⁺, *ura3-52*,
leu2-3, *trp1*, *ade2-1*, *lys2-1*, *his5*, *can1-100*) was
grown overnight at 28°C in YPD liquid medium (10 g yeast extract, 20 g bacto-peptone, 20 g dextrose per liter), and an aliquot of the culture was inoculated into 100 ml fresh YPD medium and grown until the OD₆₀₀ of the culture was 1. Cells were then collected by centrifugation and resuspended in about 200 μ l of supernatant. 40 μ l aliquots of the cell suspension were then mixed with 1-2 μ g DNA and electroporated in 2 mm-gap cuvettes using a Biorad Gene Pulser instrument set at 600 V, 200 Ω , 25 μ F. 160 μ l YPD was added and the cells were plated on selective medium containing glucose. Selective medium consisted of 6.7 g yeast nitrogen base (Difco), 0.4 g casamino acids (Difco), 0.02 g adenine sulfate, 0.03 g L-leucine, 0.02 g L-tryptophan, 0.03 g L-lysine-HCl, 0.03 g L-histidine-HCl, 2% glucose, water to 1 liter. Plates were solidified using 1.5% Difco Bacto-

agar. Transformant colonies appeared after 3 to 4 days incubation at 28°C

Expression of the *L. fenderi* hydroxylase in yeast

5 Independent transformant colonies from the previous experiment were used to inoculate 5 ml of selective medium containing either 2% glucose (gene repressed) or 2% galactose (gene induced) as the sole carbon source. Independent colonies of
10 CGY2557 transformed with pYES2 containing no insert were used as controls.

After 2 days of growth at 28°C, an aliquot of the cultures was used to inoculate 5 ml of fresh selective medium. The new culture was placed at
15 16°C and grown for 9 days.

Fatty acid analysis of yeast expressing the *L. fenderi* hydroxylase

Cells from 2.5 ml of culture were pelleted at
20 1800g, and the supernatant was aspirated as completely as possible. Pellets were then dispersed in 1 ml of 1 N methanolic HCl (Supelco, Bellafonte, PA). Transmethylation and derivatization of hydroxy fatty acids were
25 performed as described above. After drying under nitrogen, samples were redissolved in 50µl chloroform before being analyzed by GC-MS. Samples were injected into an SP2330 fused-silica

capillary column (30 m x 0.25 mm ID, 0.25 μ m film thickness, Supelco). The temperature profile was 100 - 160°C, 25°C/min, 160 - 230°C, 10°C/min, 230°C, 3 min, 230-100°C, 25°C/min. Flow rate was 0.9 ml/min. Fatty acids were analyzed using a Hewlett-Packard 5971 series Msdetector.

Gas chromatograms of derivatized fatty acid methyl esters from induced cultures of yeast containing pLesqYes contained a novel peak that eluted at 7.6 min (Figure 13). O-TMS methyl ricinoleate eluted at exactly the same position on control chromatograms. This peak was not present in cultures lacking pLesqYes or in cultures containing pLesqYes grown on glucose (repressing conditions) rather than galactose (inducing conditions). Mass spectrometry of the peak (Figure 13) revealed that the peak has the same spectrum as O-TMS methyl ricinoleate. Thus, on the basis of chromatographic retention time and mass spectrum, it was concluded that the peak corresponded to O-TMS methyl ricinoleate. The presence of ricinoleate in the transgenic yeast cultures confirms the identity of the gene as a kappa hydroxylase of this invention.

EXAMPLE 3. OBTAINING OTHER PLANT FATTY ACYL HYDROXYLASES

In a previous patent application, we

described the ways in which the castor fah12
sequence could be used to identify other kappa
hydroxylases by methods such as PCR and
heterologous hybridization. However, because of
5 the high degree of sequence similarity between $\Delta 12$
desaturases and kappa hydroxylases, prior art does
not teach how to distinguish between the two kinds
of enzymes without a functional test such as
demonstrating activity in transgenic plants or
10 another suitable host (e.g., transgenic microbial
or animal cells). The identification of the *L.*
fendleri hydroxylase provided for the development
of criteria by which a hydroxylase and a
desaturase may be distinguished solely on the
15 basis of deduced amino acid sequence information.

Figures 9A-B show a sequence alignment of the
castor and *L. fendleri* hydroxylase sequences with
the castor hydroxylase sequence and all publicly
available sequences for all plant microsomal $\Delta 12$
20 fatty acid desaturases. Of the 384 amino acid
residues in the castor hydroxylase sequence, more
than 95% are identical to the corresponding
residue in at least one of the desaturase
sequences. Therefore, none of these residues are
25 responsible for the catalytic differences between
the hydroxylase and the desaturases. Of the
remaining 16 residues in the castor hydroxylase
and 14 residues in the *Lesquerella* hydroxylase,

all but six represent instances where the
hydroxylase sequence has a conservative
substitution compared with one or more of the
desaturase sequences, or there is wide variability
5 in the amino acid at that position in the various
desaturases. By conservative, we mean that the
following amino acids are functionally equivalent:
Ser/Thr, Ile/Leu/Val/Met, Asp/Glu. Thus, these
structural differences also cannot account for the
10 catalytic differences between the desaturases and
hydroxylases. This leaves just six amino acid
residues where both the castor hydroxylase and the
Lesquerella hydroxylase differ from all of the
known desaturases and where all of the known
15 microsomal $\Delta 12$ desaturases have the identical
amino acid residue. These residues occur at
positions 69, 111, 155, 226, 304 and 331 of the
alignment in Figure 9. Therefore, these six sites
distinguish hydroxylases from desaturases. Based
20 on this analysis, we claim that any enzyme with
greater than 60% sequence identity to one of the
enzymes listed in Figure 9 can be classified as a
hydroxylase if it differs from the sequence of the
desaturases at these six positions. Because of
25 slight differences in the number of residues in a
particular protein, the numbering may vary from
protein to protein but the intent of the number
system will be evident if the protein in question

is aligned with the castor hydroxylase using the numbering system shown herein. Thus, in conjunction with the methods for using the Lesquerella hydroxylase gene to isolate homologous genes, the structural criterion disclosed here teaches how to isolate and identify plant kappa hydroxylase genes for the purpose of genetically modifying fatty acid composition as disclosed herein and in the previous application (No. 08/320,982).

In considering which of the six substitutions are solely or primarily responsible for the difference in catalytic activity of the hydroxylases of this invention and the desaturases, we consider it likely that the substitution of a Phe for a Tyr at position 226 may be solely responsible for this difference in catalytic activity because of the known participation of tyrosine radicals in enzyme catalysis. Other substitutions, such as the Ala for Ser at position 331 may have effects at modulating the overall rate of the reaction. On this basis we envision creating novel kappa hydroxylases by site directed mutagenesis of $\Delta 12$ desaturases. We also envision converting $\Delta 15$ desaturases and $\Delta 9$ desaturases to hydroxylases by similar use of site-directed mutagenesis.

EXAMPLE 4 - USING HYDROXYLASES TO ALTER THE LEVEL
OF FATTY ACID UNSATURATION

Evidence that kappa hydroxylases of this invention can be used to alter the level of fatty acid unsaturation was obtained from the analysis of transgenic plants that expressed the castor hydroxylase under control of the Cauliflower mosaic virus promoter. The construction of the plasmids and the production of transgenic Arabidopsis plants was described in Example 1 (above). The fatty acid composition of seed lipids from wild type and six transgenic lines (1-2/a, 1-2/b, 1-3/b, 4F, 7E, 7F) is shown in Table 2.

Table 2. Fatty acid composition of lipids from Arabidopsis seeds. The asterisk (*) indicates that for some of these samples, the 18:3 and 20:1 peaks overlapped on the gas chromatograph and, therefore, the total amount of these two fatty acids is reported.

Fatty acid	WT	1-2/a	1-2/b	1-3/b	4F	7E	7F
16:0	10.3	8.6	9.5	8.4	8.1	8.4	9
18:0	3.5	3.8	3.9	3.3	3.5	3.8	4.2
18:1	14.7	33	34.5	25.5	27.5	30.5	28.5
18:2	32.4	16.9	21	27.5	21.1	20.1	19.8
18:3	13.8	-	14.4	14.8	-	-	-
20:0	1.3	1.6	1	1.1	2.4	1.8	2
20:1	22.5	-	14.1	17.5	-	-	-
18:3 20:1*	-	31.2	-	-	32.1	30.8	30.6
Ricinoleic	0	0.6	0	0.1	0.2	0.7	0.9
Densipolic	0	0.6	0	0.1	0.2	0.5	0.6
Lesquerolic	0	0.2	0	0	0.2	0.2	0.6
Auricollic	0	0.1	0	0	0	0.1	0.1

The results in Table 2 show that expression of the castor hydroxylase in transgenic Arabidopsis plants caused a substantial increase in the amount of oleic acid (18:1) in the seed lipids and an approximately corresponding decrease in the amount of linoleic acid (18:2). The average amount of oleic acid in the six transgenic lines was 29.9% versus 14.7% in the wild type.

The mechanism by which expression of the castor hydroxylase gene causes increased accumulation of oleic acid is not known. An understanding of the mechanism is not required in order to exploit this invention for the directed alteration of plant lipid fatty acid composition. Furthermore, it will be recognized by one skilled in the art that many improvements of this invention may be envisioned. Of particular interest will be the use of other promoters which have high levels of seed-specific expression.

Since hydroxylated fatty acids were not detected in the seed lipids of transgenic line 1-2b, it seems likely that it is not the presence of hydroxylated fatty acids per se that causes the effect of the castor hydroxylase gene on desaturase activity. We speculate that there may be a protein-protein interaction between the hydroxylase and the $\Delta 12$ -oleate desaturase or another protein required for the overall reaction

(eg., cytochrome b5) or for the regulation of desaturase activity. We envision that the interaction between the hydroxylase and this other protein suppresses the activity of the desaturase.

5 For instance, the quaternary structure of the membrane-bound desaturases has not been established. It is possible that these enzymes are active as dimers or as multimeric complexes containing more than two subunits. Thus, if
10 dimers or multimers formed between the desaturase and the hydroxylase, the presence of the hydroxylase in the complex may disrupt the activity of the desaturase. This general hypothesis will be tested directly by the
15 production of transgenic plants in which the hydroxylase enzyme has been rendered inactive by the elimination of one or more of the histidine residues that have been proposed to bind iron molecules required for catalysis. Several of
20 these histidine residues have been shown to be essential for catalysis by site directed mutagenesis (Shanklin et al., 1994). Codons encoding histidine residues in the castor hydroxylase gene described in U.S. patent
25 application 08/320,982 will be changed to alanine residues as described by Shanklin et al. (1994). The modified genes will be introduced into transgenic plants of Arabidopsis and possibly

other species such as tobacco by the methods described in Example 1 of this application or in Example 1 of the original version of this application (U.S. application 08/320,982).

5 In order to examine the effect on all tissues, the strong constitutive cauliflower mosaic virus promoter will be used to cause transcription of the modified genes. However, it will be recognized that in order to specifically
10 examine the effect of expression of the mutant gene on seed lipids, a seed-specific promoter such as the *B. napus* napin promoter or the promoter described in Example 2, above, may be used. An expected outcome is that expression of the
15 inactive hydroxylase protein in transgenic plants will inhibit the activity of the endoplasmic reticulum-localized $\Delta 12$ -desaturase. Maximum inhibition will be obtained by expressing high levels of the mutant protein.

20 In a further embodiment of this invention, we envision that mutations that inactivate other hydroxylases, such as the *Lesquerella* hydroxylase of this invention, will also be useful for decreasing the amount of endoplasmic reticulum-
25 localized $\Delta 12$ -desaturase activity in the same way as the castor gene. In a further embodiment of this invention, we also envision that similar mutations of desaturase genes may be used to

inactivate endogenous desaturases. Thus, we envision that expression of catalytically inactive fad2 gene from Arabidopsis in transgenic Arabidopsis will inhibit the activity of the endogenous fad2 gene product.

Similarly, we envision that expression of the catalytically inactive forms of the $\Delta 12$ -desaturase from Arabidopsis or other plants in transgenic soybean in transgenic rapeseed, Crambe, *Brassica juncea*, Canola, flax, sunflower, safflower, cotton, cuphea, soybean, peanut, coconut, oil palm and corn will lead to inactivation of endogenous $\Delta 12$ -desaturase activity in these species. In a further embodiment of this invention we envision that expression of catalytically inactive forms of other desaturases such as the $\Delta 15$ -desaturases will lead to inactivation of the corresponding desaturases.

Whatever the precise basis for the inhibitory effect of the castor hydroxylase on desaturation, because the castor hydroxylase has very low nucleotide sequence homology (i.e., about 67%) to the Arabidopsis fad2 gene (encoding the endoplasmic reticulum-localized $\Delta 12$ -desaturase) we envision that the inhibitory effect of this gene, which we provisionally call "protein-mediated inhibition" ("protibition"), will have broad utility because it does not depend on a high

degree of nucleotide sequence homology between the
transgene and the endogenous target gene. In
particular, we envision that the castor
hydroxylase may be used to inhibit the endoplasmic
5 reticulum-localized $\Delta 12$ -desaturase activity of all
higher plants. Of particular relevance are those
species used for oil production. These include
but are not limited to rapeseed, Crambe, *Brassica*
juncea, Canola, flax, sunflower, safflower,
10 cotton, cuphea, soybean, peanut, coconut, oil palm
and corn.

CONCLUDING REMARKS

By the above examples, demonstration of
15 critical factors in the production of novel
hydroxylated fatty acids by expression of a kappa
hydroxylase gene from Castor in transgenic plants
is described. In addition, a complete cDNA
sequence of the *Lesquerella fendleri* kappa
20 hydroxylase is also provided. A full sequence of
the castor hydroxylase is also given with various
constructs for use in host cells. Through this
invention, one can obtain the amino acid and
nucleic acid sequences which encode plant fatty
25 acyl hydroxylases from a variety of sources and
for a variety of applications. Also revealed is a
novel method by which the level of fatty acid
desaturation can be altered in a directed way

through the use of genetically altered hydroxylase or desaturase genes.

All publications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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van de Loo, Frank
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Genetically Modified Plants
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(A) MEDIUM TYPE: Diskette, 3.50 inch, 1.44 KB storage
(B) COMPUTER: IBM PC
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(2) INFORMATION FOR SEQ ID NO:1

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 543 nucleotides
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TATTGGCACC GGCGGCACCA TTCCAACAAT GGATCCCTAG AAAAAGATGA AGTCTTTGTC 60
 CCACCTAAGA AAGCTGCAGT CANATGGTAT GTCAAATACC TCAACAACCC TCTTGGACGC 120
 ATTCTGGTGT TAACAGTTCA GTTTATCCTC GGGTGGCCTT TGTATCTAGC CTTTAATGTA 180
 TCAGGTAGAC CTTATGATGG TTTCGCTTCA CATTTCTTCC CTCATGCACC TATCTTTAAG 240
 GACCGTGAAC GTCTCCAGAT ATACATCTCA GATGCTGGTA TTCTAGCTGT CTGTTATGGT 300
 CTTTACCGTT ACGCTGCTTC ACAAGGATTG ACTGCTATGA TCTGCGTCTA CGGAGTACCG 360
 CTTTTGATAG TGAACTTTTT CCTTGTCTTG GTCACCTTCT TGCAGCACAC TCATCCTTCA 420
 TTACCTCACT ATGATTCAAC CGAGTGGGAA TGGATTAGAG GAGCTTTGGT TACGGTAGAC 480
 AGAGACTATG GAATCTTGAA CAAGGTGTTT CACAACATAA CAGACACCCA CGTAGCACAC 540
 CAC 543

(2) INFORMATION FOR SEQ ID NO:2

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 544 nucleotides
 (B) TYPE: nucleotide
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TATAGGCACC GGAGGCACCA TTCCAACACA GGATCCCTCG AAAGAGATGA AGTATTTGTC 60
 CCAAAGCAGA AATCCGCAAT CAAGTGGTAC GGCGAATACC TCAACAACCC TCCTGGTCGC 120
 ATCATGATGT TAACTGTCCA GTTCGTCTC GGATGGCCCT TGTACTTAGC CTTCAACGTT 180
 TCTGGCAGAC CCTACAATGG TTTCGCTTCC CATTTCTTCC CCAATGCTCC TATCTACAAC 240
 GACCGTGAAC GCCTCCAGAT TTACATCTCT GATGCTGGTA TTCTAGCCGT CTGTTATGGT 300
 CTTTACCGTT ACGCTGTTGC ACAAGGACTA GCCTCAATGA TCTGTCTAAA CGGAGTTCCG 360
 CTTCTGATAG TTAACTTTTT CCTCGTCTTG ATCACTTACT TACAACACAC TCACCCTGCG 420
 TTGCCTCACT ATGATTCATC AGAGTGGGAT TGGCTTAGAG GAGCTTTAGC TACTGTAGAC 480
 AGAGACTATG GAATCTTGAA CAAGGTGTTC CATAACATCA CAGACACCCA CGTCGCACAC 540

(2) INFORMATION FOR SEQ ID NO:3

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1740 nucleotides
 (B) TYPE: nucleotide
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATGAAGCTTT ATAAGAAGTT AGTTTTCTCT GGTGACAGAG AAATTNTGTC AATTGGTAGT 60
 GACAGTTGAA GCAACAGGAA CAACAAGGAT GGTGAGTGNT GATGCTGATG TGGTGATGTG 120
 TTATTCATCA AATACTAAAT ACTACATTAC TTGTTGCTGC CTAATTCTCC TATTTCTCTC 180
 GCCACCCATT TTGGACCCAC GANCCTTCCA TTAAACCCT CTCTCGTGCT ATTCACCAGA 240
 AGAGAAGCCA AGAGAGAGAG AGAGAGAATG TTCTGAGGAT CATTGTCTTC TTCATCGTTA 300
 TTAACGTAAG TTTTTTTTGA CCACTCATAT CTAAAATCTA GTACATGCAA TAGATTAATG 360
 ACTGTTCCCT CTTTGTATAT TTTCAGCTTC TTGAATTCAA GATGGGTGCT GGTGGAAGAA 420
 TAATGGTTAC CCCCTCTTCC AAGAAATCAG AACTGAAGC CCTAAAACGT GGACCATGTG 480
 AGAAACCACC ATTCACTGTT AAAGATCTGA AGAAAGCAAT CCCACAGCAT TGTTTCAAGC 540
 GCTCTATCCC TCGTTCTTTC TCCTACCTTC TCACAGATAT CACTTTAGTT TCTTGCTTCT 600
 ACTACGTTGC CACAAATTAC TTCTCTCTTC TTCCTCAGCC TCTCTCTACT TACCTAGCTT 660
 GGCCTCTCTA TTGGGTATGT CAAGGCTGTG TCTTAACCGG TATCTGGGTC ATTGGCCATG 720
 AATGTGGTCA CCATGCATTC AGTGAATATC AATGGGTAGA TGACACTGTT GGTTTTATCT 780
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 ACATCTCAGA TGCTGGTATT CTAGCTGTCT GTTATGGTCT TTACCGTTAC GCTGCTTCAC 1140
 AAGGATTGAC TGCTATGATC TCGTCTATG GAGTACCGCT TTTGATAGTG AACTTTTTTCC 1200

TTGTCTTGGT AACTTTCTTG CAGCACACTC ATCCTTCGTT ACCTCATTAT GATTCAACCG 1260
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 AGGTGTTCCA TAACATAACA GACACACATG TGGCTCATCA TCTCTTTGCA ACTATACCGC 1380
 ATTATAACGC AATGGAAGCT ACAGAGGCGA TAAAGCCAAT ACTTGGTGAT TACTACCACT 1440
 TCGATGGAAC ACCGTGGTAT GTGGCCATGT ATAGGGAAGC AAAGGAGTGT CTCTATGTAG 1500
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 AGAAGCTATG CTTTGTTTCA ATAATCTCAG AGTCCATNTA GTTGTGTTCT GGTGCATTTT 1680
 GCCTAGTTAT GTGGTGTGCG AAGTTAGTGT TCAAAGTCTG TCCTGCTGTG CTGCCCAGTG 1740
 AAGAACAAGT TTACGTGTTT AAAATACTCG GAACGAATTG ACCACAANAT ATCCAAAACC 1800
 GGCTATCCGA ATTCCATATC CGAAAACCGG ATATCCAAAT TTCCAGAGTA CTTAG 1855

2) INFORMATION FOR SEQ ID NO:4

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 384 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Gly Ala Gly Gly Arg Ile Met Val Thr Pro Ser Ser Lys Lys Ser
 Glu Thr Glu Ala Leu Lys Arg Gly Pro Cys Glu Lys Pro Pro Phe Thr
 Val Lys Asp Leu Lys Lys Ala Ile Pro Gln His Cys Phe Lys Arg Ser
 Ile Pro Arg Ser Phe Ser Tyr Leu Leu Thr Asp Ile Thr Leu Val Ser
 Cys Phe Tyr Tyr Val Ala Thr Asn Tyr Phe Ser Leu Leu Pro Gln Pro
 Leu Ser Thr Tyr Leu Ala Trp Pro Leu Tyr Trp Val Cys Gln Gly Cys

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100 110
Val Leu Thr Gly Ile Trp Val Ile Gly His Glu Cys Gly His His Ala
120
Phe Ser Asp Tyr Gln Trp Val Asp Asp Thr Val Gly Phe Ile Phe His
130 140
Ser Phe Leu Leu Val Pro Tyr Phe Ser Trp Lys Tyr Ser His Arg Arg
150 160
His His Ser Asn Asn Gly Ser Leu Glu Lys Asp Glu Val Phe Val Pro
170
Pro Lys Lys Ala Ala Val Lys Trp Tyr Val Lys Tyr Leu Asn Asn Pro
180 190
Leu Gly Arg Ile Leu Val Leu Thr Val Gln Phe Ile Leu Gly Trp Pro
200
Leu Tyr Leu Ala Phe Asn Val Ser Gly Arg Pro Tyr Asp Gly Phe Ala
210 220
Ser His Phe Phe Pro His Ala Pro Ile Phe Lys Asp Arg Glu Arg Leu
230 240
Gln Ile Tyr Ile Ser Asp Ala Gly Ile Leu Ala Val Cys Tyr Gly Leu
250
Tyr Arg Tyr Ala Ala Ser Gln Gly Leu Thr Ala Met Ile Cys Val Tyr
260 270
Gly Val Pro Leu Leu Ile Val Asn Phe Phe Leu Val Leu Val Thr Phe
280
Leu Gln His Thr His Pro Ser Leu Pro His Tyr Asp Ser Thr Glu Trp
290 300
Glu Trp Ile Arg Gly Ala Leu Val Thr Val Asp Arg Asp Tyr Gly Ile
310 320
Leu Asn Lys Val Phe His Asn Ile Thr Asp Thr His Val Ala His His
330
Leu Phe Ala Thr Ile Pro His Tyr Asn Ala Met Glu Ala Thr Glu Ala
340 350
Ile Lys Pro Ile Leu Gly Asp Tyr Tyr His Phe Asp Gly Thr Pro Trp
360
Tyr Val Ala Met Tyr Arg Glu Ala Lys Glu Cys Leu Tyr Val Glu Pro
370 380
Asp Thr Glu Arg Gly Lys Lys Gly Val Tyr Tyr Tyr Asn Asn Lys Leu

(2) INFORMATION FOR SEQ ID NO:5

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 387 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Gly Gly Gly Gly Arg Met Ser Thr Val Ile Thr Ser Asn Asn Ser
Glu Lys Lys Gly Gly Ser Ser His Leu Lys Arg Ala Pro His Thr Lys
Pro Pro Phe Thr Leu Gly Asp Leu Lys Arg Ala Ile Pro Pro His Cys
Phe Glu Arg Ser Phe Val Arg Ser Phe Ser Tyr Val Ala Tyr Asp Val
Cys Leu Ser Phe Leu Phe Tyr Ser Ile Ala Thr Asn Phe Phe Pro Tyr
Ile Ser Ser Pro Leu Ser Tyr Val Ala Trp Leu Val Tyr Trp Leu Phe
Gln Gly Cys Ile Leu Thr Gly Leu Trp Val Ile Gly His Glu Cys Gly
His His Ala Phe Ser Glu Tyr Gln Leu Ala Asp Asp Ile Val Gly Leu
Ile Val His Ser Ala Leu Leu Val Pro Tyr Phe Ser Trp Lys Tyr Ser
His Arg Arg His His Ser Asn Ile Gly Ser Leu Glu Arg Asp Glu Val
Phe Val Pro Lys Ser Lys Ser Lys Ile Ser Trp Tyr Ser Lys Tyr Ser
Asn Asn Pro Pro Gly Arg Val Leu Thr Leu Ala Ala Thr Leu Leu Leu
Gly Trp Pro Leu Tyr Leu Ala Phe Asn Val Ser Gly Arg Pro Tyr Asp
Arg Phe Ala Cys His Tyr Asp Pro Tyr Gly Pro Ile Phe Ser Glu Arg

230 240
 Glu Arg Leu Gln Ile Tyr Ile Ala Asp Leu Gly Ile Phe Ala Thr Thr
 250
 Phe Val Leu Tyr Gln Ala Thr Met Ala Lys Gly Leu Ala Trp Val Met
 260 270
 Arg Ile Tyr Gly Val Pro Leu Leu Ile Val Asn Cys Phe Leu Val Met
 280
 Ile Thr Tyr Leu Gln His Thr His Pro Ala Ile Pro Arg Tyr Gly Ser
 290 300
 Ser Glu Trp Asp Trp Leu Arg Gly Ala Met Val Thr Val Asp Arg Asp
 310 320
 Tyr Gly Val Leu Asn Lys Val Phe His Asn Ile Ala Asp Thr His Val
 330
 Ala His His Leu Phe Ala Thr Val Pro His Tyr His Ala Met Glu Ala
 340 350
 Thr Lys Ala Ile Lys Pro Ile Met Gly Glu Tyr Tyr Arg Tyr Asp Gly
 360
 Thr Pro Phe Tyr Lys Ala Leu Trp Arg Glu Ala Lys Glu Cys Leu Phe
 370 380
 Val Glu Pro Asp Glu Gly Ala Pro Thr Gln Gly Val Phe Trp Tyr Arg
 Asn Lys Tyr

09885189 062101

(2) INFORMATION FOR SEQ ID NO:6

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 383 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

10
 Met Gly Ala Gly Gly Arg Met Pro Val Pro Thr Ser Ser Lys Lys Ser
 20 30
 Glu Thr Asp Thr Thr Lys Arg Val Pro Cys Glu Lys Pro Pro Phe Ser

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40
Val Gly Asp Leu Lys Lys Ala Ile Pro Pro His Cys Phe Lys Arg Ser
50 60
Ile Pro Arg Ser Phe Ser Tyr Leu Ile Ser Asp Ile Ile Ile Ala Ser
70 80
Cys Phe Tyr Tyr Val Ala Thr Asn Tyr Phe Ser Leu Leu Pro Gln Pro
90
Leu Ser Tyr Leu Ala Trp Pro Leu Tyr Trp Ala Cys Gln Gly Cys Val
100 110
Leu Thr Gly Ile Trp Val Ile Ala His Glu Cys Gly His His Ala Phe
120
Ser Asp Tyr Gln Trp Leu Asp Asp Thr Val Gly Leu Ile Phe His Ser
130 140
Phe Leu Leu Val Pro Tyr Phe Ser Trp Lys Tyr Ser His Arg Arg His
150 160
His Ser Asn Thr Gly Ser Leu Glu Arg Asp Glu Val Phe Val Pro Lys
170
Gln Lys Ser Ala Ile Lys Trp Tyr Gly Lys Tyr Leu Asn Asn Pro Leu
180 190
Gly Arg Ile Met Met Leu Thr Val Gln Phe Val Leu Gly Trp Pro Leu
200
Tyr Leu Ala Phe Asn Val Ser Gly Arg Pro Tyr Asp Gly Phe Ala Cys
210 220
His Phe Phe Pro Asn Ala Pro Ile Tyr Asn Asp Arg Glu Arg Leu Gln
230 240
Ile Tyr Leu Ser Asp Ala Gly Ile Leu Ala Val Cys Phe Gly Leu Tyr
250
Arg Tyr Ala Ala Ala Gln Gly Met Ala Ser Met Ile Cys Leu Tyr Gly
260 270
Val Pro Leu Leu Ile Val Asn Ala Phe Leu Val Leu Ile Thr Tyr Leu
280
Gln His Thr His Pro Ser Leu Pro His Tyr Asp Ser Ser Glu Trp Asp
290 300
Trp Leu Arg Gly Ala Leu Ala Thr Val Asp Arg Asp Tyr Gly Ile Leu
310 320
Asn Lys Val Phe His Asn Ile Thr Asp Thr His Val Ala His His Leu

330
 Phe Ser Thr Met Pro His Tyr Asn Ala Met Glu Ala Thr Lys Ala Ile
 340 350
 Lys Pro Ile Leu Gly Asp Tyr Tyr Gln Phe Asp Gly Thr Pro Trp Tyr
 360
 Val Ala Met Tyr Arg Glu Ala Lys Glu Cys Ile Tyr Val Glu Pro Asp
 370 380
 Arg Glu Gly Asp Lys Lys Gly Val Tyr Trp Tyr Asn Asn Lys Leu

(2) INFORMATION FOR SEQ ID NO:7

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 384 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

10
 Met Gly Ala Gly Gly Arg Met Gln Val Ser Pro Pro Ser Lys Lys Ser
 20 30
 Glu Thr Asp Asn Ile Lys Arg Val Pro Cys Glu Thr Pro Pro Phe Thr
 40
 Val Gly Glu Leu Lys Lys Ala Ile Pro Pro His Cys Phe Lys Arg Ser
 50 60
 Ile Pro Arg Ser Phe Ser His Leu Ile Trp Asp Ile Ile Ile Ala Ser
 70 80
 Cys Phe Tyr Tyr Val Ala Thr Thr Tyr Phe Pro Leu Leu Pro Asn Pro
 90
 Leu Ser Tyr Phe Ala Trp Pro Leu Tyr Trp Ala Cys Gln Gly Cys Val
 100 110
 Leu Thr Gly Val Trp Val Ile Ala His Glu Cys Gly His Ala Ala Phe
 120
 Ser Asp Tyr Gln Trp Leu Asp Asp Thr Val Gly Leu Ile Phe His Ser
 130 140
 Phe Leu Leu Val Pro Tyr Phe Ser Trp Lys Tyr Ser His Arg Arg His

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

10
Ser Leu Leu Thr Ser Phe Ser Tyr Val Val Tyr Asp Leu Ser Phe Ala
20
Phe Ile Phe Tyr Ile Ala Thr Thr Tyr Phe His Leu Leu Pro Gln Pro
30
Phe Ser Leu Ile Ala Trp Pro Ile Tyr Trp Val Leu Gln Gly Cys Leu
40
Leu Thr Arg Val Cys Gly His His Ala Phe Ser Lys Tyr Gln Trp Val
50
Asp Asp Val Val Gly Leu Thr Leu His Ser Thr Leu Leu Val Pro Tyr
60
Phe Ser Trp Lys Ile Ser His Arg Arg His His Ser Asn Thr Gly Ser
70
Leu Asp Arg Asp Glu Arg Val Lys Val Ala Trp Phe Ser Lys Tyr Leu
80
Asn Asn Pro Leu Gly Arg Ala Val Ser Leu Leu Val Thr Leu Thr Ile
90
Gly Trp Pro Met Tyr Leu Ala Phe Asn Val Ser Gly Arg Pro Tyr Asp
100
Ser Phe Ala Ser His Tyr His Pro Tyr Arg Val Arg Leu Leu Ile Tyr
110
Val Ser Asp Val Ala Leu Phe Ser Val Thr Tyr Ser Leu Tyr Arg Val
120
Ala Thr Leu Lys Gly Leu Val Trp Leu Leu Cys Val Tyr Gly Val Pro
130
Leu Leu Ile Val Asn Gly Phe Leu Val Thr Ile Thr Tyr Leu Arg Val
140
His Tyr Asp Ser Ser Glu Trp Asp Trp Leu Lys Gly Ala Leu Ala Thr
150
Met Asp Arg Asp Tyr Gly Ile Leu Asn Lys Val Phe His His Ile Thr
160
Asp Thr His Val Ala His His Leu Phe Ser Thr Met Pro His Tyr His
170
Leu Arg Val Lys Pro Ile Leu Gly Glu Tyr Tyr Gln Phe Asp Asp Thr
180
260
270

Pro Phe Tyr Lys Ala Leu Trp Arg Glu Ala Arg Glu Cys Leu Tyr Val
 280
 Glu Pro Asp Glu Gly Thr Ser Glu Lys Gly Val Tyr Trp Tyr Arg Asn
 290 300
 Lys Tyr Leu Arg Val

(2) INFORMATION FOR SEQ ID NO:9

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 302 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Phe Ser Tyr Val Val Tyr Asp Leu Thr Ile Ala Phe Cys Leu Tyr Tyr
 10
 Val Ala Thr His Tyr Phe His Leu Leu Pro Gly Pro Leu Ser Phe Arg
 20 30
 Gly Met Ala Ile Tyr Trp Ala Val Gln Gly Cys Ile Leu Thr Gly Val
 40
 Trp Val Val Ala Phe Ser Asp Tyr Gln Leu Leu Asp Asp Ile Val Gly
 50 60
 Leu Ile Leu His Ser Ala Leu Leu Val Pro Tyr Phe Ser Trp Lys Tyr
 70 80
 Ser His Arg Arg His His Ser Asn Thr Gly Ser Leu Glu Arg Asp Glu
 90
 Val Phe Val Pro Lys Val Ser Lys Tyr Leu Asn Asn Pro Pro Gly Arg
 100 110
 Val Leu Thr Leu Ala Val Thr Leu Thr Leu Gly Trp Pro Leu Tyr Leu
 120
 Ala Leu Asn Val Ser Gly Arg Pro Tyr Asp Arg Phe Ala Cys His Tyr
 130 140
 Asp Pro Tyr Gly Pro Ile Tyr Ser Val Ile Ser Asp Ala Gly Val Leu
 150 160
 Ala Val Val Tyr Gly Leu Phe Arg Leu Ala Met Ala Lys Gly Leu Ala
 170

180
 Trp Val Val Cys Val Tyr Gly Val Pro Leu Leu Val Val Asn Gly Phe
 190
 200
 Leu Val Leu Ile Thr Phe Leu Gln His Thr His Val Ser Glu Trp Asp
 210
 Trp Leu Arg Gly Ala Leu Ala Thr Val Asp Arg Asp Tyr Gly Ile Leu
 220
 230
 Asn Lys Val Phe His Asn Ile Thr Asp Thr His Val Ala His His Leu
 240
 250
 Phe Ser Thr Met Pro His Tyr His Ala Met Glu Ala Thr Val Glu Tyr
 260
 Tyr Arg Phe Asp Glu Thr Pro Phe Val Lys Ala Met Trp Arg Glu Ala
 270
 280
 Arg Glu Cys Ile Tyr Val Glu Pro Asp Gln Ser Thr Glu Ser Lys Gly
 290
 Val Phe Trp Tyr Asn Asn Lys Leu Ala Met Glu Ala Thr Val
 300

(2) INFORMATION FOR SEQ ID NO:10

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 372 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

10
 Met Gly Ala Gly Gly Arg Met Thr Glu Lys Glu Arg Glu Lys Gln Glu
 20
 Gln Leu Ala Arg Ala Thr Gly Gly Ala Ala Met Gln Arg Ser Pro Val
 30
 40
 Glu Lys Pro Pro Phe Thr Leu Gly Gln Ile Lys Lys Ala Ile Pro Pro
 50
 His Cys Phe Glu Arg Ser Val Leu Lys Ser Phe Ser Tyr Val Val His
 60
 70
 Asp Leu Val Ile Ala Ala Ala Leu Leu Tyr Phe Ala Leu Ala Ile Ile
 80
 90
 Pro Ala Leu Pro Ser Pro Leu Arg Tyr Ala Ala Trp Pro Leu Tyr Trp

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100
Ile Ala Gln Gly Ala Phe Ser Asp Tyr Ser Leu Leu Asp Asp Val Val
110
120
Gly Leu Val Leu His Ser Ser Leu Met Val Pro Tyr Phe Ser Trp Lys
130
140
Tyr Ser His Arg Arg His His Ser Asn Thr Gly Ser Leu Glu Arg Asp
150
160
Glu Val Phe Val Pro Lys Lys Lys Glu Ala Leu Pro Trp Tyr Thr Pro
170
Tyr Val Tyr Asn Asn Pro Val Gly Arg Val Val His Ile Val Val Gln
180
190
Leu Thr Leu Gly Trp Pro Leu Tyr Leu Ala Thr Asn Ala Ser Gly Arg
200
Pro Tyr Pro Arg Phe Ala Cys His Phe Asp Pro Tyr Gly Pro Ile Tyr
210
220
Asn Asp Arg Glu Arg Ala Gln Ile Phe Val Ser Asp Ala Gly Val Val
230
240
Ala Val Ala Phe Gly Leu Tyr Lys Leu Ala Ala Ala Phe Gly Val Trp
250
Trp Val Val Arg Val Tyr Ala Val Pro Leu Leu Ile Val Asn Ala Trp
260
270
Leu Val Leu Ile Thr Tyr Leu Gln His Thr His Pro Ser Leu Pro His
280
Tyr Asp Ser Ser Glu Trp Asp Trp Leu Arg Gly Ala Leu Ala Thr Met
290
300
Asp Arg Asp Tyr Gly Ile Leu Asn Arg Val Phe His Asn Ile Thr Asp
310
320
Thr His Val Ala His His Leu Phe Ser Thr Met Pro His Tyr His Ala
330
Met Glu Ala Thr Lys Ala Ile Arg Pro Ile Leu Gly Asp Tyr Tyr His
340
350
Phe Asp Pro Thr Pro Val Ala Lys Ala Thr Trp Arg Glu Ala Gly Glu
360
Cys Ile Tyr Val Glu Pro Glu Asp Arg Lys Gly Val Phe Trp Tyr Asn
370
Lys Lys Phe Xaa

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(2) INFORMATION FOR SEQ ID NO:11

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 224 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Trp Val Met Ala His Asp Cys Gly His His Ala Phe Ser Asp Tyr Gln
Leu Leu Asp Asp Val Val Gly Leu Ile Leu His Ser Cys Leu Leu Val
Pro Tyr Phe Ser Trp Lys His Ser His Arg Arg His His Ser Asn Thr
Gly Ser Leu Glu Arg Asp Glu Val Phe Val Pro Lys Lys Lys Ser Ser
Ile Arg Trp Tyr Ser Lys Tyr Leu Asn Asn Pro Pro Gly Arg Ile Met
Thr Ile Ala Val Thr Leu Ser Leu Gly Trp Pro Leu Tyr Leu Ala Phe
Asn Val Ser Gly Arg Pro Tyr Asp Arg Phe Ala Cys His Tyr Asp Pro
Tyr Gly Pro Ile Tyr Asn Asp Arg Glu Arg Ile Glu Ile Phe Ile Ser
Asp Ala Gly Val Leu Ala Val Thr Phe Gly Leu Tyr Gln Leu Ala Ile
Ala Lys Gly Leu Ala Trp Val Val Cys Val Tyr Gly Val Pro Leu Leu
Val Val Asn Ser Phe Leu Val Leu Ile Thr Phe Leu Gln His Thr His
Pro Ala Leu Pro His Tyr Asp Ser Ser Glu Trp Asp Trp Leu Arg Gly
Ala Leu Ala Thr Val Asp Arg Asp Tyr Gly Ile Leu Asn Lys Val Phe
His Asn Ile Thr Asp Thr Gln Val Ala His His Leu Phe Thr Met Pro

(2) INFORMATION FOR SEQ ID NO:12

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 nucleotides
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCTCTTTTGT GCGCTCATTC

20

(2) INFORMATION FOR SEQ ID NO:13

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 nucleotides
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CGGTACCAGA AAACGCCTTG

20

(2) INFORMATION FOR SEQ ID NO:14

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 nucleotides
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TAYWSNCAYM GNMGNCA YCA

20

(2) INFORMATION FOR SEQ ID NO:15

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 nucleotides
- (B) TYPE: nucleotide
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

RTGRTGNGCN ACRTGNGTRT C

21